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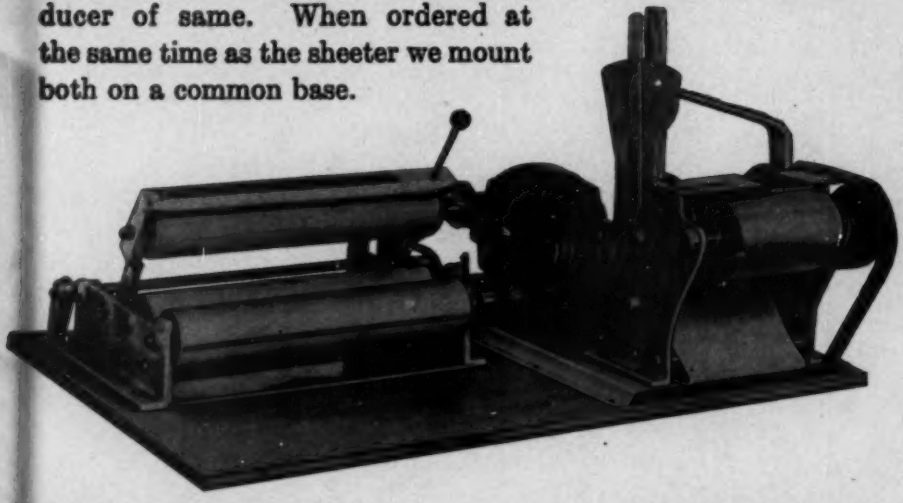

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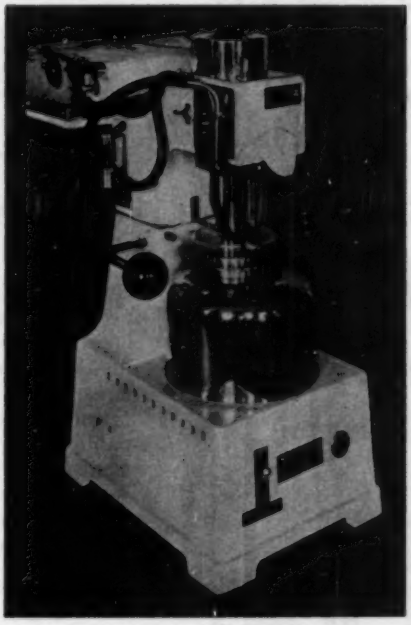

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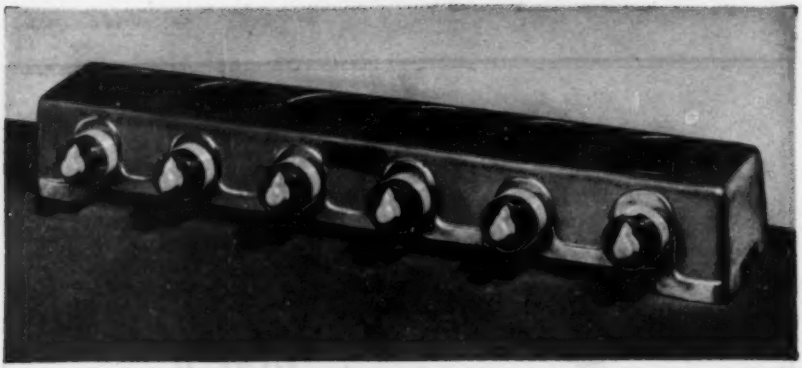

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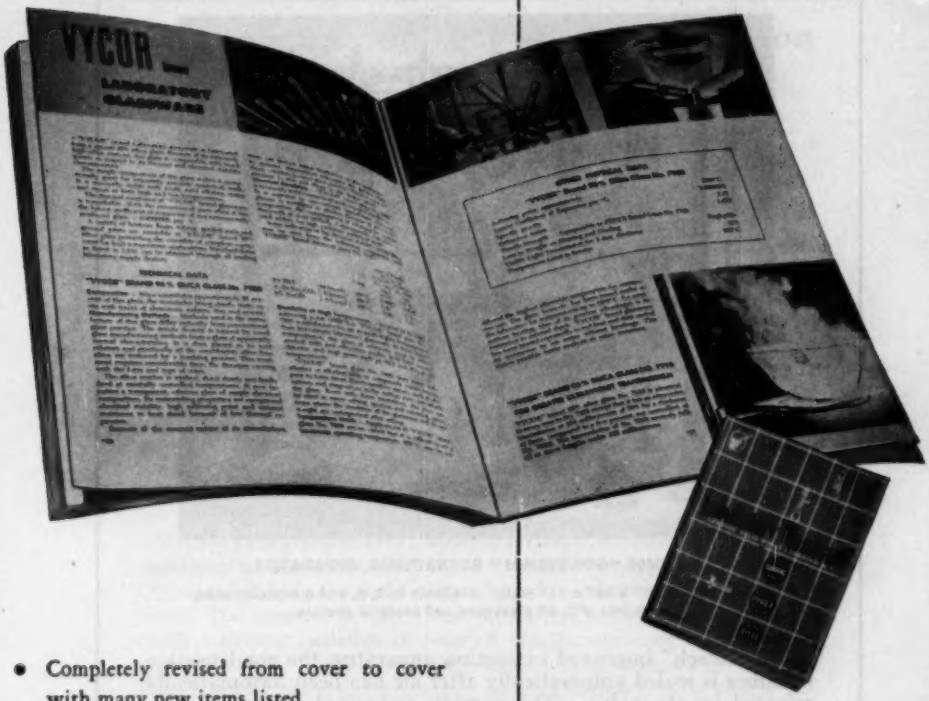

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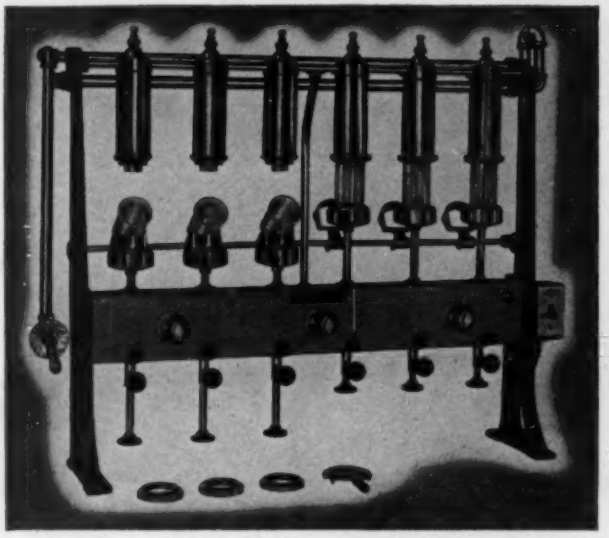

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CEREAL CHEMISTRY

VOL. XXVIII NOVEMBER, 1951 NO. 6

A TITRATION METHOD FOR THE DETERMINATION OF CATECHOL OXIDASE ACTIVITY IN DRY PLANT TISSUES [1]

MAX MILNER

ABSTRACT

A titration method is described for the determination of catechol oxidation by crude dry materials such as wheat bran and seeds. The ground tissue is agitated with catechol and excess ascorbic acid in the presence of oxygen in such a manner that the oxidation product, *o*-benzoquinone, is continually reduced by the ascorbic acid to catechol. The extent of catechol oxidation at the end of a fixed incubation period then is determined by titrating the residual ascorbic acid with 2,6-dichlorophenolindophenol. Oxidase activity can be expressed in terms of either the amount of catechol or ascorbic acid oxidized. Omission of catechol from the reaction mixture makes possible the determination of ascorbic acid oxidase, and preliminary tests showed insignificant amounts of this enzyme in wheat.

Optimum pH of the buffering medium for catechol oxidase activity in wheat bran was found to be 5.2 with citrate buffer, and the optimum pH for stability of ascorbic acid to destruction by oxygen was also close to this value. Optimum levels for catechol and ascorbic acid concentration were shown to exist. The presence in wheat and other crop seeds of a non-enzymatic catalyst causing oxidation of catechol was demonstrated by boiling the ground material. Metaphosphoric acid-acetic acid solution when used as a protein precipitant was shown to stop catechol oxidase activity almost completely, and also to inhibit markedly the oxidation of ascorbic acid. Similar effects were not produced by tungstic acid in the presence of either acetic or sulfuric acid solutions or by trichloroacetic acid.

The method here described was applied in the determination of the catechol oxidase activity of different varieties of several common crop seeds.

In the course of studies on the activity of various oxidizing enzymes in maturing wheat, lack of an exact method to determine catechol oxidase (tyrosinase) activity was encountered although wheat bran long has been known as an excellent source of the enzyme (4). Preliminary attempts to prepare active extracts of the enzyme by ordinary means (grinding in buffer, autolytic digestion and extraction, etc.) in order to assay the enzyme by the chronometric method of Miller

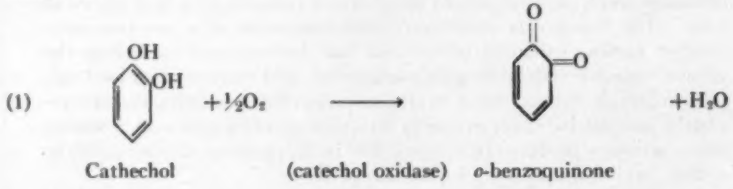
[1] Manuscript received March 19, 1951. Contribution No. 180, Department of Milling Industry, Kansas Agricultural Experiment Station, Manhattan, Kansas.

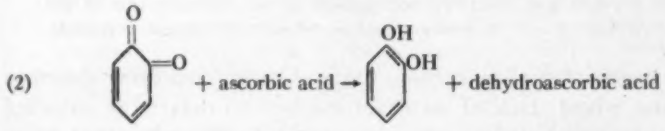
and Dawson (6) failed to incorporate sufficient soluble enzyme to show any demonstrable activity. Failure to obtain an active extract of the dry tissue also ruled out measurements by traditional manometric means (2) or by a recently suggested leuco dye oxidation procedure (8). Effort was then directed toward the development of a method using the ground dry tissue as an enzyme source.[2]

It has been known for some time that ascorbic acid apparently inhibits melanin pigment formation due to tyrosinase action (1). This phenomenon appears to be the basis for the use of ascorbic acid to prevent darkening of frozen fruits in storage (9, 11). In the present studies, this property of ascorbic acid provided the basis for a quantitative method for the determination of catechol oxidase in dry materials such as wheat bran and seeds.

Principle of Method

The fact that catechol oxidase of wheat bran was not readily extractable for quantitative determination suggested that a method might be devised whereby a suitable substrate such as catechol could be induced to diffuse into the tissue and there react with the enzyme, and that the extent of the reaction might be determined by chemical means. In this connection it has been reported (6) that ascorbic acid reacts with *o*-benzoquinone, the product of the enzymic oxidation of catechol, and reduces it quantitatively to catechol. As long as any ascorbic acid remains, this reaction continues in the following manner:





These facts suggested the possibility of determining catechol oxidase by using an excess of ascorbic acid in a reaction mixture composed principally of enzyme, catechol, and ascorbic acid. The ascorbic acid remaining after the reaction has been allowed to proceed

[2] This paper was completed prior to the publication of the method of Wallerstein *et al.* (10) for the determination of pyrogallol oxidase activity in brewing materials such as rice, wheat, and corn which utilizes homogenates to produce oxidation products which may be estimated colorimetrically.

for a suitable time interval can then be determined by suitable quantitative methods such as that using 2,6-dichlorophenolindophenol (5).

It is of interest that when the studies reported here were virtually completed, Asimov and Dawson (3) reported that they had employed a similar method, using potassium iodate for the determination of ascorbic acid, to study the reaction inactivation of purified tyrosinase extracts obtained from the common mushroom.

In the preliminary work in the present studies it was found that continuous agitation of the reaction mixture containing dry bran as an enzyme source, in a flask open to the air, caused an increase in the rate of reaction, and that shaking in the presence of oxygen increased the reaction rate even more. The importance of a high oxygen tension for the reaction was established and the empirical method described in the following section was developed for wheat bran and is obviously applicable to any dry plant tissue.

Materials

Equipment. Major equipment required is a water thermostat (25°C.), fitted with a shaking mechanism capable of accommodating a number of 125 ml. Erlenmeyer flasks. The flasks are connected in a gas train by means of rubber stoppers and glass and rubber tubing, and during the reaction period oxygen from a cylinder of compressed oxygen is passed through the train continually at the rate of a few bubbles per second. It is unnecessary that the oxygen bubble through the reaction mixture since the agitation, if properly controlled, will cause it to be sufficiently aerated.

Enzyme Source. Any finely ground or dispersed plant tissue is suitable. When different plant tissues are to be compared these must be ground to a comparable fineness and the same weight of sample must be used for all determinations. In the present studies grinding was carried out by means of the intermediate Wiley mill equipped with a No. 40 sieve.

Solutions

Citrate-Phosphate Buffer of pH 5.2. One liter 0.2 *M* citric acid and 1-l. 0.4 *M* sodium orthophosphate (mono-hydrogen) are made up and mixed in the ratio required to give pH 5.2 ± 0.1. A citric acid-sodium hydroxide-hydrochloric acid-mixture of the same pH may be used alternatively.

Catechol Solution 1%. Dissolve 5.0 g. reagent grade catechol in distilled water and make up to 500 ml. Prepare fresh daily.

Ascorbic Acid 1%. Dissolve exactly 2.5 g. reagent grade ascorbic acid in distilled water and dilute to 250 ml. Prepare fresh daily.

Metaphosphoric Acid-Acetic Acid Mixture. In a 2-l. beaker mix 80 ml. glacial acetic acid with 800 ml. of distilled water. Dissolve in this mixture, preferably with constant stirring or shaking, 30 g. metaphosphoric acid crystals. Dilute to 1-l. Filter through a fluted filter or a Buchner funnel with suction. Store in the refrigerator.

Indophenol Solution. Dissolve 0.1 g. sodium bicarbonate and 0.25 g. 2,6-dichlorophenolindophenol (sodium 2,6-dichlorobenzenone-indophenol, Eastman) in about 100 ml. of warm distilled water, cool, and dilute to 500 ml. For standardization, dilute 5 ml. of 1% ascorbic acid solution to 50 ml. with the metaphosphoric-acetic acid mixture and titrate a 10 ml. aliquot of this solution (10 mg. ascorbic acid) with indophenol solution to the end point and calculate the dye strength in terms of milligrams ascorbic acid per milliliter of dye. The unused portion of the dye solution may be stored in the refrigerator for use within one week but should be standardized daily against fresh ascorbic acid solution.

Method

Weigh into a 125-ml. Erlenmeyer flask a sample of enzyme-containing material (± 0.01 g.) of a size which preliminary trial has shown will cause the destruction of not more than half of the ascorbic acid present in the reaction mixture. To the flask add the following reagents in the order named: 5 ml. water, 25 ml. citrate-phosphate buffer (pH 5.2), 10 ml. 1% ascorbic acid solution, and 10 ml. 1% catechol.

Immediately clamp the flask in the shaker mechanism of the 25°C. thermostat, connect the flasks in a gas train, turn on a slow stream of oxygen, start the shaker, and allow the reaction to proceed for one hour.

After precisely one hour stop the reaction by adding to each flask 50 ml. metaphosphoric acid-acetic acid mixture from a fast-flowing pipet. Remove the flask from the water bath and shake. Filter the reaction mixture through a Whatman No. 4 or equivalent porous paper. Pipet a 10 ml. aliquot of the clear reaction filtrate into a 50 ml. Erlenmeyer flask and titrate to the end point with standard indophenol solution. This titration value is termed "A" in the calculation of activity. A small drop of octyl alcohol was found useful to prevent the solution from frothing during titration. The apparent oxidation of ascorbic acid due to *o*-benzoquinone produced from catechol by enzyme action must be corrected by the amount of non-enzymic destruction of ascorbic acid due to auto-oxidation. For this reason an enzyme blank determination, in which all reagents are used and the enzyme is omitted, is conducted with the reaction determination. This titre is termed "B" in the calculation of activity. Evidence was

obtained that under certain circumstances it is desirable to deduct the activity of a control sample which has been boiled for 15 minutes to destroy the enzyme activity.

When using wheat bran, or similar dry materials, no correction was found to be necessary for ascorbic acid oxidase activity, since the amount of this enzyme in such tissues was found to be extremely small.

Enzyme activity may be expressed in terms of milligrams ascorbic acid destroyed per gram of crude dry enzyme per hour, and can be calculated from the following values:

A = ml. indophenol required to titrate the 10 ml. aliquot of enzyme activity determination.

B = ml. indophenol required to titrate the 10 ml. aliquot of an enzyme-free blank.

C = ascorbic acid equivalent (in mg.) of 1 ml. of standard indophenol solution.

D = dilution factor, to convert activity value obtained in reaction aliquot to activity value for entire reaction mixture. Thus, where a 10 ml. aliquot of a total of 100 ml. of the digest is titrated, $D = \frac{100}{10} = 10$.

With these values, the activity of the crude material in terms of mg. ascorbic acid destroyed per gram per hour would be:

$$\frac{(B - A) \times C \times D}{\text{sample weight}}.$$

Experimental Variables Affecting the Method

Oxygen. The critical necessity of high oxygen tension for maximum tyrosinase activity was demonstrated early in the preliminary work in an experiment designed to investigate the effect of increasing aeration and gas availability by shaking with air and with added oxygen, as well as the influence of such treatment on the auto-oxidation of ascorbic acid. Five grams of pulverized wheat bran was used as the enzyme source. The results of this study are presented in Table I. The data of Table I indicate clearly that aeration by shaking in the presence of air only was insufficient to supply the oxygen necessary to assure maximum activity. Thus when air alone was applied with shaking, as in reaction flask No. 5, the reaction rate was considerably greater than when air or oxygen was in contact with a stationary reaction mixture (flasks 1 and 2). The value recorded for flask No. 6 is an underestimation of the influence of shaking in the presence of oxygen since all of the ascorbic acid originally present was used up

before the end of the reaction period. These results also indicate that considerable ascorbic destruction occurs due to non-enzymatic oxidation (flask No. 3). Enzyme activity values for flasks Nos. 4, 5, and 6 and for all activity values reported in subsequent experiments have been corrected for this ascorbic acid blank value.

pH. In a preliminary investigation of the influence of pH on the activity of the catechol oxidase in dry wheat bran, it was realized that pH not only influences the enzyme activity but also has a marked effect on the destruction of ascorbic acid by oxygen. The extent of this auto-oxidation was determined in the enzyme-free reaction blank.

Determination of the influence of variations in pH were carried out with both the citric acid-dibasic sodium phosphate mixtures (referred to as phosphate buffer) and the citric acid-sodium hydroxide-hydrochloric acid mixture (referred to as citrate buffer). The catechol oxidase values of a sample of wheat bran in terms of milligrams of

TABLE I

INFLUENCE OF SHAKING IN THE PRESENCE OF AIR AND OF OXYGEN ON ASCORBIC ACID DESTRUCTION AND ON CATECHOL OXIDASE ACTIVITY

Reaction Flask No.	*Experimental Conditions*	*Mg. Ascorbic Acid Destroyed per Hr.*
1	Ascorbic acid, stationary contact with O_2	2.8
2	Ascorbic acid, shaken in contact with air	3.8
3	Ascorbic acid, shaken in contact with O_2	5.5
4	Enzyme and ascorbic acid, stationary contact with O_2	5.3
5	Enzyme and ascorbic acid, shaken in contact with air	8.9
6	Enzyme and ascorbic acid, shaken in contact with O_2	20.0

ascorbic acid oxidized per gram of bran per hour, in both the citrate and phosphate buffers in the pH range of 2.4 to 6.8, appear in Fig. 1, together with curves showing the influence of the various pH values on the extent of auto-oxidation of ascorbic acid in the blanks.

Reference to Fig. 1 indicates that the catechol oxidase activity of wheat bran is at a maximum under the conditions imposed, at pH 5.2 with citrate buffer and at about 4.7 with phosphate buffer. Activity in the citrate buffer was somewhat greater than in the phosphate buffer, but this is apparently partially due to the somewhat greater stability of ascorbic acid to auto-oxidation in the citrate buffer. It is of interest that the stability of ascorbic acid to auto-oxidation as indicated by the plots of the blank determinations decreases with

increasing pH values above 2.4 until a value of about pH 4.5. A zone of minimum oxidation appears, centered at about pH 5.2 with both citrate and phosphate buffer, although the level of destruction is appreciably greater in the latter. pH values higher than this range are accompanied by marked increases in the destruction of ascorbic acid in phosphate buffer but only minor increases in oxidation were encountered with citrate buffer. The citrate buffer obviously protects ascorbic acid from oxidation in this range of pH to a greater extent than does the phosphate. Preliminary studies with acetate buffer in

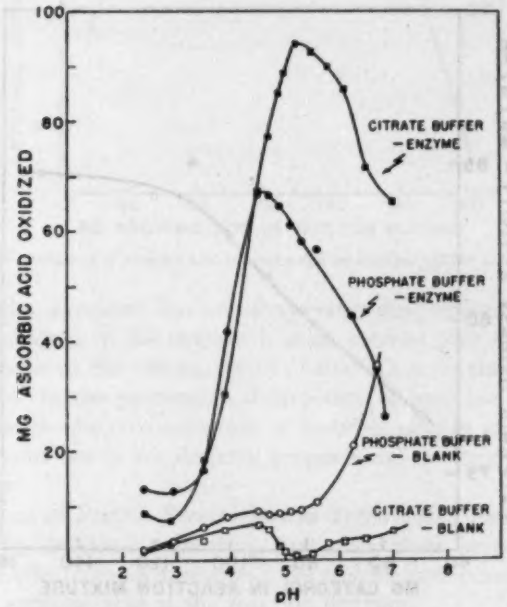


FIG. 1. Influence of pH on oxidation of ascorbic acid by catechol oxidase of wheat bran (mg./g./hr.) and on auto-oxidation of ascrobic acid in blanks.

this pH range indicated a very marked destruction of ascorbic acid in the presence of acetate ion.

Influence of Catechol Concentration. Although catechol concentration in the enzyme digest remains, theoretically, at a constant level throughout the reaction period because of reduction by ascorbic acid of the *o*-benzoquinone formed by enzyme action, it was of interest, nevertheless, to determine if an optimum concentration of catechol existed. In this experiment the effect of eight concentrations of catechol in the reaction mixture (50 to 150 mg.) was studied. The experimental results shown in Fig. 2 indicate that catechol concentrations in the

reaction mixture below 100 mg. limit the enzyme action. With a tissue such as wheat bran, the substrate must diffuse into the tissue before enzyme action can occur. This process is probably slower than the enzyme action itself so that it is to be expected that a high concentration of the substrate would be necessary to offset the rate characteristics of the diffusion process. It is of interest that the catechol appeared to have very little influence on the non-enzymic oxidation of ascorbic acid since the blank values showed little variation as the catechol concentration was increased.

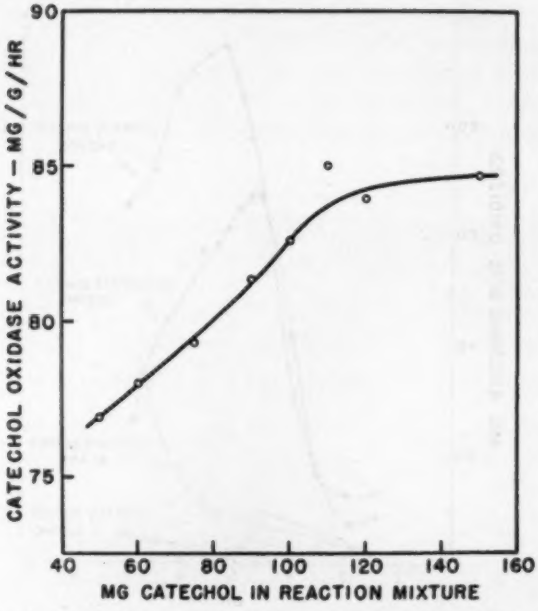


FIG. 2. Influence of catechol concentration on catechol oxidase activity.

Influence of Ascorbic Acid Concentration. Optimum concentration of ascorbic acid was determined in a manner similar to that employed with catechol using bran as an enzyme source. Ascorbic acid concentration in the reaction mixture between 50 and 160 mg. yielded the results shown graphically in Fig. 3.

Reference to Fig. 3 indicates that a rapid increase in enzyme activity occurs with increasing concentrations of ascorbic acid up to 80 mg. but that beyond 120 mg. a decline in activity appears. This suggests an inhibitory effect of high concentrations of ascorbic acid on the enzyme.

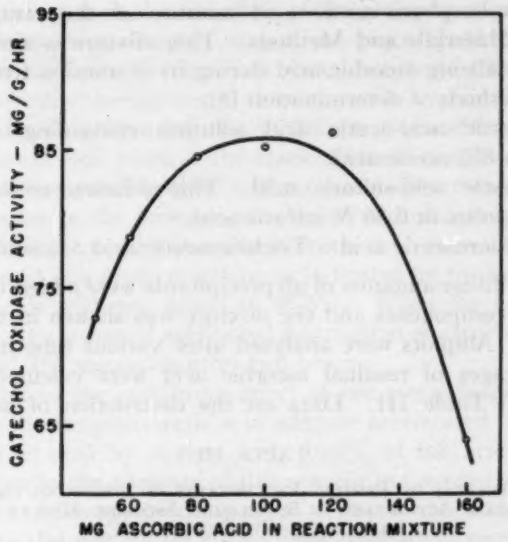


FIG. 3. Influence of ascorbic acid concentration on catechol oxidase activity.

The results obtained for non-enzymatic destruction of ascorbic acid, as determined in the enzyme blanks, showed that except for an anomalous value at the 120-mg. level (Table II), a regular and almost linear increase in the percentage destruction of ascorbic acid due to oxygen occurs as the concentration of ascorbic acid in increased, but that this destruction is not directly proportional to the concentration of this reagent.

Effectiveness of Protein Precipitants in Terminating Enzyme Action. The following protein-inactivating materials were investigated for their relative effectiveness in stopping the enzyme action and stabilizing the residual ascorbic acid of the reaction mixture:

TABLE II

RELATIONSHIP OF INITIAL CONCENTRATION OF ASCORBIC ACID TO PERCENTAGE DESTRUCTION OF THE REAGENT BY OXYGEN IN ONE HOUR

Ascrobic Acid	
Initial Concentration	*Per Cent Destroyed*
mg./100 ml.	
50	6.6
60	7.3
80	8.1
100	9.4
120	8.3
160	15.0

A. Metaphosphoric-acetic acid mixture of the composition indicated in Materials and Methods. This mixture is similar to that used for stabilizing ascorbic acid during its extraction from tissues in standard methods of determination (5).

B. Tungstic acid-acetic acid solution containing 3% sodium tungstate in 8% acetic acid.

C. Tungstic acid-sulfuric acid. This solution contained 1.2% sodium tungstate in 0.36 *N* sulfuric acid.

D. Trichloroacetic acid. Trichloroacetic acid 5% solution.

Fifty milliliter amounts of all precipitants were added to the 50 ml. of reaction components and the mixture was shaken in the presence of oxygen. Aliquots were analyzed after various time intervals and the percentages of residual ascorbic acid were calculated and are indicated in Table III. Data on the destruction of ascorbic acid

TABLE III

EFFECTIVENESS OF PROTEIN PRECIPITANTS IN INHIBITING CATECHOL OXIDASE ACTION AND IN STABILIZING ASCORBIC ACID IN THE PRESENCE OF OXYGEN AT 25°C.

Reaction Components Present[1]	Residual Ascorbic Acid in Presence of Various Precipitants after Different Time Intervals in Minutes (Per Cent)			
	0	30	60	120
Metaphosphoric Acid—Acetic Acid Precipitant				
Ascorbic acid only	100.0	97.8	94.9	87.7
Ascorbic acid and precipitant	100.0	99.8	99.8	99.5
Ascorbic acid, precipitant, and enzyme	100.0	98.0	98.3	97.5
Tungstic Acid—Acetic Acid Precipitant				
Ascorbic acid only	100.0	91.4	89.3	85.6
Ascorbic acid and precipitant	100.0	82.0	82.0	76.9
Ascorbic acid, precipitant, and enzyme	100.0	83.3	78.5	74.3
Tungstic Acid—Sulfuric Acid Precipitant				
Ascorbic acid only	100.0	94.1	91.5	84.5
Ascorbic acid and precipitant	100.0	98.8	98.7	98.8
Ascorbic acid, precipitant, and enzyme	100.0	94.5	94.1	91.9
Trichloroacetic Acid Precipitant				
Ascorbic acid only	100.0	90.0	88.9	80.1
Ascorbic acid and precipitant	100.0	87.4	70.5	46.8
Ascorbic acid, precipitant, and enzyme	100.0	87.9	71.8	49.4

[1] In addition to catechol and phosphate buffer.

when present alone, and also the effect of the precipitant in protecting it from the destructive effects of oxygen, are given (Table III).

The data of Table III indicate metaphosphoric-acetic acid to be the most effective precipitant of those tested in terminating enzyme action and also in preventing the oxidation of ascorbic acid. The data show that in four trials, in the absence of any precipitant, 80.1% to 87.7% of the original ascorbic acid remained after two hours when shaken in oxygen in the presence of catechol and phosphate buffer at pH 5.2. Addition of metaphosphoric-acetic acid mixture stabilized the ascorbic acid in a marked fashion as indicated by the fact that the residual ascorbic acid was raised to 99.5%. When the enzyme was present, a slight amount of additional destruction of ascorbic acid was noted, only 97.5% remaining after two hours.

In contrast to the stabilizing effect of the metaphosphoric-acetic acid mixture the tungstic-acetic acid mixture accelerated the destruction of ascorbic acid by oxygen, only 76.9% of the acid remaining after two hours in the presence of this reagent in contrast with 85.7% in the absence of the precipitating agent. Furthermore, the tungstic-acetic mixture did not inhibit the enzyme completely since a further destruction of 2.6% ascorbic acid occurred when wheat bran was present.

Marked improvement in ascorbic acid stabilization was secured by substituting sulfuric acid for acetic acid in the tungstic acid reagent. Residual values for ascorbic acid then attained similar levels to those with metaphosphoric-acetic acid (98.8%). The tungstic-sulfuric acid mixture, however, did not terminate enzyme action satisfactorily as indicated by the considerably lower ascorbic acid concentration which remained after two hours when the enzyme was present (91.9%).

Of all reagents tested trichloroacetic acid appeared to be the most destructive of ascorbic acid. After two hours in the presence of this protein precipitant the ascorbic acid concentration dropped to 46.8% of the amount initially present, and with the enzyme in the mixture to 49.4%, indicating that the crude enzyme material actually mitigated the destructive effect of the trichloroacetic acid.

These results suggest that acetic acid is highly conducive to the destruction of ascorbic acid by oxygen unless the phosphate ion is present.

Influence of Boiling on Catechol Oxidation. It was of interest to determine whether non-enzymatic catalysts are present in dry plant tissues such as wheat bran which would contribute to the apparent enzymatic activity. For this study, the bran sample was suspended in buffer and boiled under a reflux condenser for 15 minutes, a treatment which preliminary studies had shown to produce maximum

enzyme inactivation. After cooling, the ascorbic acid and catechol components of the normal reaction mixture were added and the trial was completed in the usual manner. The results of the study appear in Table IV which shows also the influence of boiling on the blank as well as the total catechol oxidation activity of the sample.

These results indicate that a small but appreciable amount of the total catechol oxidation activity in wheat bran is apparently due to non-enzymatic catalysts. Thus a boiled control determination would appear to be necessary if it is desired to know the extent of strictly enzymatic catechol oxidation. That this may not be strictly the case is indicated in the data of the following section.

Application of the Method to Various Seed Species. The utility of the method described for the determination of catechol oxidase activity in crude wheat bran was demonstrated in an investigation of 12 common kinds of crop seeds as indicated in Table V, where data are presented for the amount of catechol oxidation in the boiled control, as well as the enzymatic activity when corrected for this control and the ascorbic acid blank. These were clean, viable seeds obtained from stocks of the Agronomy Department of Kansas State College. The only preparation of the seeds prior to catechol oxidase determination was grinding, as well as boiling in the case of determination of non-enzymatic activity of the control. Reference to Table V indicates that wheat possesses the highest concentration of catechol oxidase activity of all cereals examined, followed closely by oats. Of the other cereals, barley, kafir and yellow corn contain lesser amounts, while white corn and sorghum contain very little of the enzyme when correction for the blank only is considered. Soybeans were relatively high in catechol oxidase activity but flaxseed, also an oil-bearing seed, contained only a slight amount. The data for white corn and for all three varieties of sorghum tested are striking in that slightly greater catechol oxidation was exhibited by the boiled controls than was shown by the unheated

TABLE IV

OXIDATION OF CATECHOL BY BOILED AND UNBOILED SAMPLES OF WHEAT BRAN

Experimental Conditions	*Rate of Catechol Oxidation*[1]
Blank, unheated	0.3
Blank, boiled 15 min., cooled	0.3
Control, bran sample boiled in buffer 15 min., cooled	19.0
Active, bran sample unheated	129.2

[1] Milligrams ascorbic acid oxidized in 1 hour by 1 g. of material.

TABLE V

OXIDATION OF CATECHOL BY SOME COMMON CROP SEEDS

Seed Crop and Variety	Activity of Boiled Control[1]	Catechol Oxidase Activity[1]	
		Corrected for Ascorbic Acid Blank	Corrected for Boiled Control
Barley, Beecher	6.7	11.1	6.1
Barley, Reno	6.5	7.7	3.4
Corn, white (hybrid)	3.7	0.2	—
Corn, yellow (hybrid)	4.4	6.4	3.7
Flaxseed (petroleum ether extracted)	7.0	6.5	1.2
Kafir, red	3.9	7.0	3.8
Oats, Nemaha	5.3	19.2	15.7
Sorghum, Atlas (white)	4.9	1.7	—
Sorghum, Schrock (brown)	5.5	3.7	—
Sorghum, Westland (yellow)	4.4	1.9	—
Soybeans, Wabash	7.0	13.7	8.4
Wheat, Pawnee	6.2	28.0	23.5

[1] Milligrams ascorbic acid oxidized in 1 hour by 1 g. of material.

material. Repeated experiments showed this effect to be valid and indicates the formation of non-enzymatic catalysts in these cereals by heat. It is also to be noted that since these determinations were made on the entire seed, the results do not take into account the probability that the enzyme is concentrated in the outer tissues of the grain and that accordingly the relative plumpness of the various seeds would have a diluting effect on the enzyme content.

Discussion

The method here described is useful as a survey and control method when the catechol oxidase enzyme can not be extracted quantitatively by normal means. For studies involving enzyme preparations of high purity which may be subject to rapid inactivation, methods which allow for the accurate determination of initial reaction velocities, such as the modified chronometric procedure of Miller, Mallette, Roth and Dawson (7) and the colorimetric method of Smith and Stotz (8) might prove more suitable. The method described here, however, is unique in that it involves diffusion of the substrate to the enzyme in the intact plant tissue and will thus show quantitatively differences in the distribution of the enzyme in various natural plant materials.

The method is also applicable to the determination of ascorbic acid oxidase, the only major change being the elimination of catechol from the reaction mixture. Such an application has not been perfected but data obtained using this procedure on ground wheat and bran indicated that the concentration of the enzyme in such materials in comparison to that of catechol oxidase is insignificant. However,

wheat bran was shown to have a small amount of non-enzymatic catalytic activity for the oxidation of catechol when the material was boiled, which is manifested by the oxidation of ascorbic acid.

The data concerning the stability of ascorbic acid to oxidation under various conditions are of interest beyond their pertinence to this study. The marked stabilizing effect which citrate ions have upon ascorbic acid under oxidative conditions and the lesser effect of the phosphate ion is clearly indicated. In contrast, acids such as acetic and trichloroacetic enhance its oxidative destruction. The finding of an optimum pH zone for the stability of ascorbic acid to oxidation (pH 5.2 for citrate buffer) is also of interest.

The small increase in catechol oxidation obtained by boiling certain cereals which contain virtually no catechol oxidase suggests that a true estimation of enzymatic activity in grains high in catechol oxidase such as wheat, may not require correction for a boiled control. This phenomenon of increased catechol oxidation in certain cereals due to boiling is the subject of further investigation.

Acknowledgments

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EFFECT OF MONOGLYCERIDES ON STARCH, FLOUR, AND BREAD: A MICROSCOPIC AND CHEMICAL STUDY [1,2,3]

E. J. STRANDINE, G. T. CARLIN, G. A. WERNER, AND R. P. HOPPER

ABSTRACT

Small quantities of monoglycerides in shortenings decrease the swelling or hydration of the starch granule and inhibit the release of water-soluble starch (chiefly amylose). Monoglyceride shortenings increase the softness of bread and decrease the rate of crumb firming upon storage as measured by means of a modified Bloom Gelometer. These phenomena are attributed to the effect of the monoglyceride on the starch fraction of flour.

Settling and centrifuging experiments on starch, flour, and bread slurries made with and without monoglycerides demonstrated that these shortenings decrease the rate of swelling and gelatinization of the starch granule. This effect was verified by microscopic studies of the starch granules in the slurries. Monoglyceride shortening used in starch or flour slurries decreased the amount of released soluble starch as demonstrated by the intensity of the blue color developed through addition of Lugol's solution to cooked starch slurries. The presence of monoglyceride shortenings appeared to inhibit the development of the familiar blue color which is due to iodine-amylose reaction. The decrease in soluble starch was verified further by measuring the amount of soluble starch which could be precipitated by ethyl alcohol from the slurries in the various experiments. The substitution of water-soluble starch for 5% of the flour in bread increased the firmness of the bread. The hydration or swelling of gluten was only slightly affected by the presence of monoglyceride shortening. However, the gluten strands in bread made with monoglyceride shortening were finer in texture than in other breads.

These studies indicate that the striking differences in firmness produced by monoglyceride shortenings in bread may be due to their effect in decreasing the swelling and water imbibition of the starch during the baking process. Such decreased imbibition would automatically make available a larger portion of the dough water for more complete gluten hydration. Monoglyceride shortenings also prevent the release of the water-soluble starch fraction from the starch granules, thus keeping this tough cementing substance in discontinuous aggregates and preventing its release on to the gluten strands and around the starch granules.

Shortenings used in bakery goods may contain varying amounts of mono- and diglycerides, depending upon the method of manufacture and the formulation. The monoglyceride content may range from a few tenths of 1% in freshly rendered lard to around 40% or 60% in

[1] Manuscript received April 26, 1950.
[2] Contribution from Swift & Company, Research Laboratories, Chicago, Illinois.
[3] Data in this paper presented at U. S. Food & Drug Administration, Bread Standards Hearing November, 1948, and at the annual meeting of the American Association of Cereal Chemists, May 1949.

some of the superglycerinated shortenings. Although it is common knowledge that monoglycerides affect the characteristics of bakery products, very little is known relative to their mode of action. The purpose of the present paper is to report the results of microscopic and chemical experiments on the effects of monoglycerides on starch, gluten, flour, and bread.

Included in this work were sedimentation studies of starch and wheat flour gels, colorimetric observations of the iodine-starch reaction, determination of soluble starch after precipitation, firmness ratings of bread slices, and microscopic observations of starch and bread.

Several workers (1, 2, 14, 19) have used the microscopic approach in the study of dough and bread thus supplementing the chemical findings of other investigators. Sjostrom (17) has investigated the starches microscopically under varying experimental conditions and recorded his observations by means of a series of excellent photomicrographs. He has described the peculiar disintegration of wheat starch granules when heated to temperatures near the boiling point.

Hopper (7), and Thomas (18) have discussed the role of monoglyceride shortenings with reference to the physical characteristics of bread. Quantitative measurements of the shearing strength of bread slices, and of the tensile strength of loaf sections clearly indicate the increase in tenderness due to the use of these shortenings. Favor and Johnson (3) have reported that polyoxyethylene monostearate retards the development of firmness of bread crumb with age by retarding the gelation of the starch. Lord (12) has concluded that polyoxyethylene monostearate has a shortening action in bread which softens bread crumb by depressing the swelling of starch.

Materials and Methods

One of the standard methods used in studying the hydration or swelling of starch is the determination of the sedimentation volume (13). To accentuate any effects that might be produced, dilute slurries of starch, gluten, or flour with high proportions of shortening and water were employed. In the first series of tests 4 g. of commercial wheat starch,[4] 96 g. of distilled water, and 0.8 g. of various shortening combinations,[5] as shown in Table I, were heated to various temperatures with constant stirring. After heating, the slurries were transferred immediately to graduated cylinders and allowed to stand over-

[4] The wheat starch used in these experiments represented commercial Canadian wheat starch prepared by International Grain Milling Company, London, Ontario, Canada.

[5] The monoglyceride shortenings used in these tests contained about 9.0% monoglyceride (Swift & Company's Tri-Co) in one case and about 40% monoglyceride (Swift & Company's Esterine) in a second, while the lard was fresh prime steam rendered lard containing 0.50% naturally occurring monoglyceride.

night. The volume of the sedimented starch was then determined and expressed as the percentage of the total volume of the slurry.

The heated starch slurries were examined microscopically and photomicrographs were taken to show the degree of hydration and gelatinization of the starch granules. After the volume of sedimented starch had been determined, the slurry was remixed, a few drops of Lugol's (potassium iodide-iodine) solution added, and the resulting color noted.

In other tests similarly proportioned slurries of starch, water, and shortening were heated, allowed to cool, and then centrifuged. The supernatant liquid was poured into three volumes of absolute ethyl alcohol. This caused precipitation of the "soluble starch" fraction which was then centrifuged to determine its volume.

The effect of monoglyceride shortening on the swelling of gluten and flour was also investigated. Bread and dough as well as slurries of wheat starch, gluten, and flour mixed with or without monoglycerides were examined under the microscope directly either with normal illumination or with polarized light. In mixing the doughs, 1% of Fast Green was used to color the protein and 1% of Sudan IV was used to color the fat or shortening. In many cases, especially in dough and bread, direct examination was not very practical and a standard histological technique involving fixing, dehydration, and paraffin embedding was employed. This technique was satisfactory for a study of the gluten and starch but could not be used to study fat distribution, due to the solvent action of the solutions used in preparing embedded samples. After embedding, the paraffin-embedded dough or bread could be sliced on a rotary microtome to a thickness of 10 μ. Photomicrographs of samples of starch slurries, dough, and bread were taken.

Fat distribution in dough and bread was determined by direct observation of samples made with colored fat. Another technique in studying fat globules was to agitate a small piece of bread or dough in a drop of cold water on a glass slide. The cold water congealed the fat in the shape and size in which it was present in the sample. Under the microscope, these microscopic fat masses could be measured and counted.

An adaptation of the Bloom Gelometer was used to test the firmness of bread, by measuring the weight necessary to depress a circular plunger (25 mm. diameter) 4 mm. into a slice of bread 48 mm. in thickness (17).

Results

Studies with Wheat Starch Slurries. The volume of starch which could be settled from wheat starch slurries after heating to various

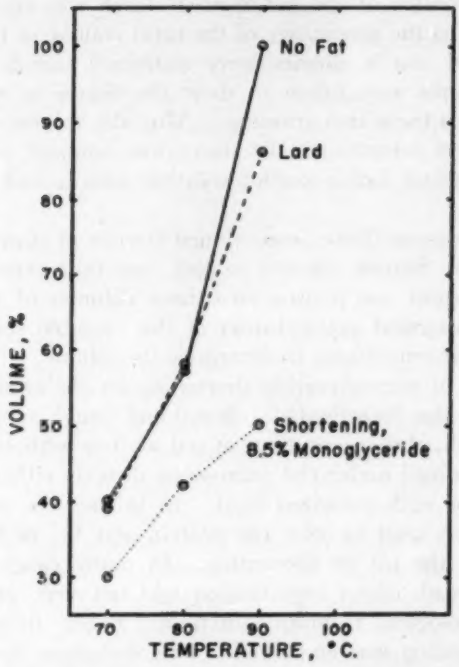

FIG. 1. Effect of monoglyceride shortening on the hydration of wheat starch as determined by sedimentation measurements. Volume of sedimented starch expressed as per cent of total slurry volume.

temperatures was decisively influenced by the kind and amount of shortening used in the preparation of the slurry. These data are shown graphically in Fig. 1. The presence of monoglycerides decreased the volume of the settled starch indicating that the monoglycerides had inhibited the swelling or hydration of the starch granules.

The reaction of the cooled starch slurries to a few drops of dilute Lugol's solution varied considerably, ranging from yellow, to pink, to violet, to blue depending upon the presence or absence of monoglycerides and the relative proportions of soluble amylose and amylopectin. The slurry with lard or no fat produced a blue color and the slurries with the monoglyceride a reddish violet to light yellow color. Although various interpretations may be given to explain the variations in color, microscopic examination of the iodine-treated slurries revealed a dark blue granular fraction and a pink-staining fraction. The blue color may have been due to the reaction of the iodine with the released amylose fraction while the reddish color was probably due to the amylopectin fraction of the wheat starch.

The red-stained shortening could be observed as globules of various sizes dispersed between the swollen starch granules. No attempts to measure and count these globules between the starch granules were made, due to the fact that much of the fat floated to the top of the sedimentation tubes. Less fat on top of sedimentation tubes was observed in the slurries made with monoglyceride.

Microscopic examination of the sedimented starch revealed that, during heating, the starch granules had reached various stages of hydration or gelatinization depending upon the temperature to which the slurry had been heated and on the presence or absence of monoglycerides. When heated in water, the starch granules underwent a progressive series of changes which involved loss of birefringence, swelling, collapse, or rupture. For purposes of clarity these may be designated as stages 1, 2, 3, respectively. In stage 1 the starch granules increased in size only slightly but lost their birefringence. The swollen starch granules (stage 2) appeared to consist of several concentric layers of denser material surrounding an inner granular material (Fig. 3). In the final stage of hydration (stage 3) the swollen starch granules either collapsed and assumed a saddle-shaped structure or they appeared ruptured. The collapsed denser outer material stained pink with dilute Lugol's solution and probably consisted chiefly of amylopectin while the released granular material stained dark blue and was probably the amylose fraction.

The stage of gelatinization or hydration of the starch differed in the various slurries. When the starch slurries made without shortening or with lard were heated to 80°C. (Fig. 2), most of the starch granules had reached the stage of hydration where the starch vesicles had collapsed to the saddle-shaped structure or had ruptured (stage 3), while at this temperature most of the starch granules in the slurry made with monoglyceride shortening were swollen (stage 2) and had not yet collapsed. The presence of monoglyceride delayed or in-

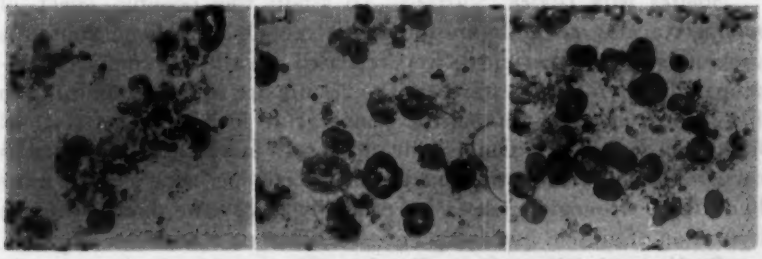

FIG. 2. Photomicrographs showing the effect of lard and monoglyceride on the swelling of wheat starch heated in 4% aqueous slurries to 80°C. *Left:* Wheat starch slurry. *Center:* Wheat starch slurry containing 0.8% lard. *Right:* Wheat starch slurry containing 0.8% monoglyceride shortening (9.0% monoglyceride content). Magnification approx. 100 ×.

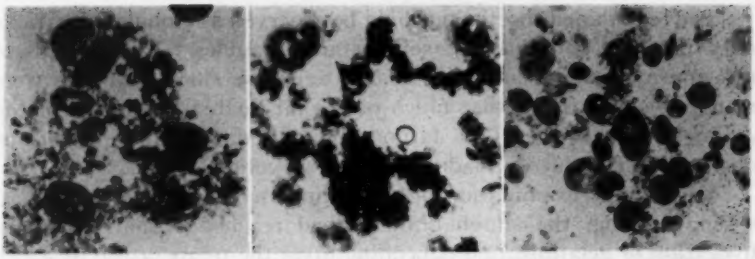

FIG. 3. Photomicrographs showing the effect of lard and monoglyceride on the swelling of wheat starch heated in 4% aqueous slurries to 90°C. *Left:* Wheat starch slurry. *Center:* Wheat starch slurry containing 0.8% lard. *Right:* Wheat starch slurry containing 0.8% monoglyceride shortening (9.0% monoglyceride content). Magnification approx. 100 ×.

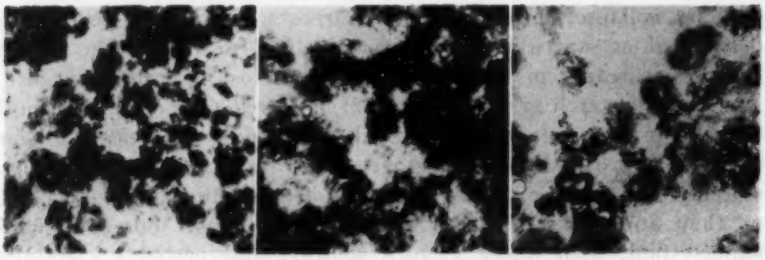

FIG. 4. Photomicrographs showing the effect of lard and monoglyceride on the swelling of wheat starch heated in 4% aqueous slurries to 98°C. *Left:* Wheat starch slurry. *Center:* Wheat starch slurry containing 0.8% lard. *Right:* Wheat starch slurry containing 0.8% monoglyceride shortening (9.0% monoglyceride content). Magnification approx. 100 ×.

hibited the hydration of the starch. At 90°C., all the starch granules in slurries made with lard or without shortening had collapsed (stage 3) while only a few of the starch granules in slurries made with monoglyceride shortening reached this stage (Fig. 3). When heated to 98°C. (Fig. 4) the starch granules in all three experiments had reached stage 3. When treated with Lugol's iodine solution, particles outside of the starch granules stained an intense blue, while the collapsed starch granules stained pink.

Studies with Gluten Slurries. To study the possible effect of monoglycerides on the hydration of the protein fraction of flour, the previous settling experiments were repeated substituting commercially-prepared gluten for the starch. The results are shown graphically in Fig. 5 and indicate that as the temperature increased, the state of hydration increased. Only slight differences in swelling or hydration occurred, the gluten slurries made with shortening being slightly more hydrated. Monoglycerides very probably have little direct effect on hydration of gluten, but exert a greater effect on starch.

Studies with Wheat Flour Slurries. The combined wheat starch-gluten swelling effect was studied by means of 4% flour-water slurries

containing 0.8 g. of various shortenings. These slurries were heated to various temperatures, allowed to settle overnight, and the volume of the sediment ascertained. The presence of monoglyceride shortenings reduced the swelling of the flour when heated in dilute aqueous slurries.

To determine the effect of flour concentration, the sedimentation experiments were repeated using 10% flour and 90% water. Fat-soluble dye (Sudan IV) was used to color the fat and define more sharply the interface of the flour-fat gel and supernatant liquid centrifuged from the mass. The resulting mixtures after heating were so viscous that centrifuging was necessary to achieve sedimentation of the hydrated flour (Fig. 7). The results of sedimentation tests were similar to those of the previous experiments in which 4% flour was used, again demonstrating that monoglycerides inhibited the swelling of the starch granules. The reaction of the slurries of flour to Lugol's solution was similar to that of the starch slurries. Those containing lard or no shortening gave a blue color while those with monoglyceride gave a reddish-violet to yellow color. Microscopic examination of

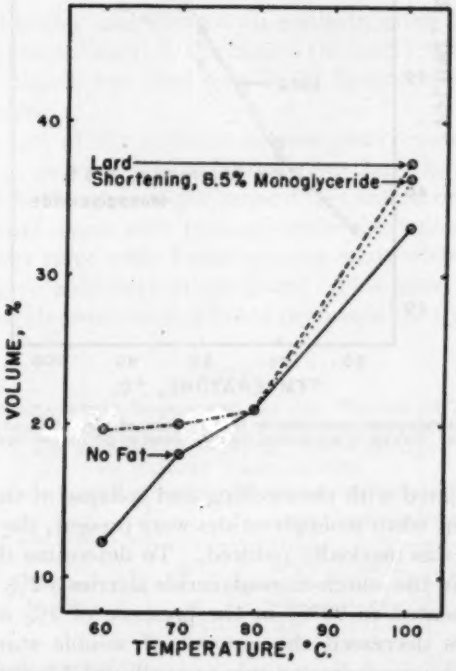

FIG. 5. Effect of monoglyceride shortening on the hydration of wheat gluten as determined by sedimentation measurements. Volume of sedimented gluten expressed as per cent of total slurry volume.

these flour slurries revealed that the presence of monoglycerides inhibited or delayed the swelling and collapse of the starch granules.

Absolute ethyl alcohol was used to precipitate water-soluble material contained in the supernatant liquid of the centrifuged flour slurry. The precipitate stained a deep blue color with Lugol's iodine solution, was readily soluble in water and was probably a fraction containing amylose. These results (Table I) show that the amount of the soluble starch fraction (chiefly amylose) increased with tempera-

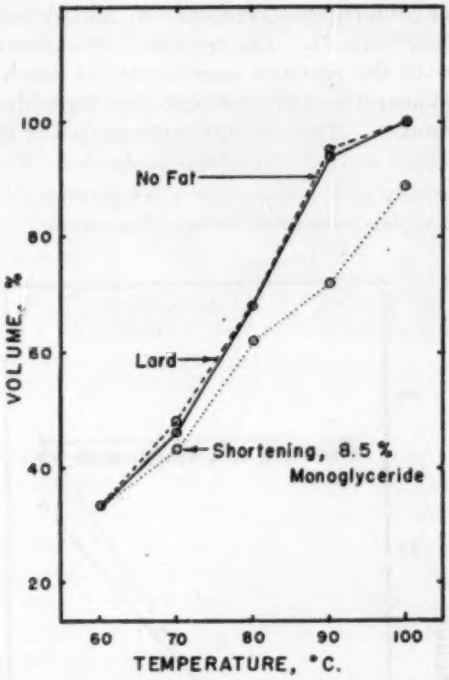

FIG. 6. Effect of monoglyceride shortening on the hydration of wheat flour as determined by sedimentation studies. Volume of sedimented flour expressed as per cent of total slurry volume.

ture and correlated with the swelling and collapse of the starch granules. However, when monoglycerides were present, the volume of the soluble starch was markedly reduced. To determine the fate of this soluble starch in the starch-monoglyceride slurries a 2% soluble starch solution was heated to 90°C. in the presence of 2% monoglyceride. Monoglycerides decreased the amount of soluble starch in starch-water systems by precipitating this normally soluble fraction.

Experiments with Bread. Slurries of bread and water were prepared

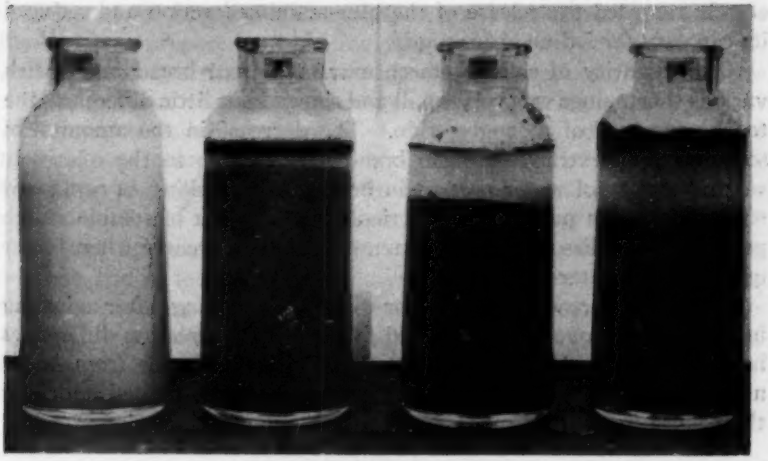

FIG. 7. Centrifuged flour mixtures (10%) after heating to 90°C. No. 1 without shortening, No. 2 with lard, No. 3 with monoglyceride shortening (8% monoglyceride content) and No. 4 with monoglyceride shortening (10% monoglyceride content).

in a Waring Blendor and allowed to settle in graduated cylinders. The volume of the sediment in the case of the bread made with monoglyceride was slightly less than that of the bread made with lard or without shortening.

Water extracts of the different breads when treated with dilute Lugol's solution gave various color reactions but these were not as pronounced as in the case of the more dilute starch or flour slurries. Extracts of bread made with monoglyceride shortening gave a light pink to chocolate color while bread extracts made without shortening or with lard gave a distinct purple color. This again demonstrated that monoglyceride shortening in bread decreased the hydration of the

TABLE I

EFFECT OF MONOGLYCERIDE SHORTENING ON THE VOLUME OF WATER-SOLUBLE BUT ETHYL ALCOHOL-INSOLUBLE FRACTION EXTRACTED FROM A 4% WHEAT FLOUR SLURRY AFTER HEATING TO VARIOUS TEMPERATURES

Slurry (4 g. Flour + 96 g. H_2O)	Wet Centrifuged Volume				
	60°C.	70°C.	80°C.	90°C.	98°C.
	ml.	*ml.*	*ml.*	*ml.*	*ml.*
0.0 g. fat added	0.4	2.0	2.4	4.4	4.4
0.8 g. lard added	0.4	1.6	3.2	4.4	4.0
0.8 g. monoglyceride shortening added (9.0% monoglyceride)	0.4	0.4	0.4	0.8	0.8

starch, retarded the release of the blue-staining fraction and reduced its capacity for iodine absorption.

The quantity of soluble starch extracted from bread made with various shortenings was very small and showed but little difference due to the presence of monoglyceride. The decrease in the amounts of soluble starch extractable from bread may be due to the relatively small quantity of water present in bread during baking as compared to that found in more dilute slurries. The amount of soluble starch produced and dissolved at high temperatures is greater when larger quantities of water are present.

Samples of bread, made with or without shortening, after agitating in water to remove the starch and fat, showed interesting differences in the gluten framework (Fig. 8). The gluten strands from bread made with monoglyceride shortening were more subdivided and finer than those from bread made with lard or without shortening.

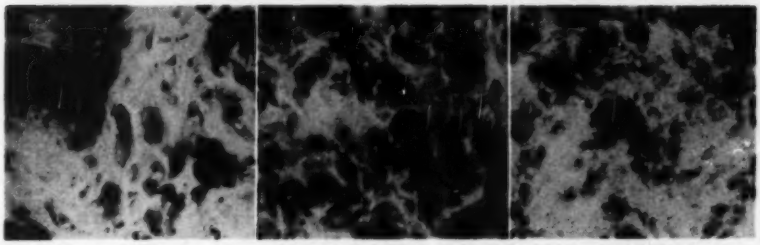

FIG. 8. *Left:* Gluten network in bread made without shortening. *Center:* With lard. *Right:* With 40% monoglyceride. Magnification approx. 5 ×.

Microscopic examination of slurries of bread made with or without monoglyceride shortenings has shown that the starch granules swell and lose their birefringence during baking but that very little collapse of starch granules occurs, due to the relatively small amount of moisture present in the dough. Limited and preliminary size-frequency studies of starch granules removed from bread by agitating bread in water indicated that more of the starch granules swell to a larger size in breads made either without shortening or with lard than in the breads made with monoglyceride shortenings. The results of a typical count based on one thousand starch granules indicated that 74% of the starch granules in bread made without shortening had diameters of 15 μ or less, while 85% of the starch granules in bread made with monoglyceride shortening were 15 μ or less in diameter. Further counts of this type would be desirable.

In another set of experiments ethyl alcohol-insoluble starch (chiefly amylose) precipitated from a water extraction of starch was

substituted for 5% of the flour used in making doughs. This starch fraction was prepared by heating wheat starch and water to 98°C., cooling, and centrifuging to obtain the supernatant liquid. The soluble fraction was precipitated by mixing the supernatant liquid with three volumes of absolute ethyl alcohol. The alcohol precipitated material was centrifuged, dried, and finely ground. In making the bread, no discernible differences in water absorption, makeup characteristics or loaf volume were observed.

The firmness of this bread was compared with other bread made without added soluble starch (amylose), using the modified Bloom Gelometer. Results of two different tests (2 loaves each) shown in Table II are based on firmness readings of 12 slices from each loaf of bread.

TABLE II

EFFECT OF AMYLOSE ON FIRMNESS OF BREAD

Test Breads	Firmness Index[1]	
	Av. of Test 1	Av. of Test 2
Lard and flour	1.00	1.00
Lard and "amylose" substituted for 5% of flour	[2]	0.97
Monoglyceride shortening and flour	0.77	0.80
Monoglyceride shortening and "amylose" substituted for 5% of flour	0.89	0.90

[1] Frimness index $= \frac{\text{firmness of test loaf}}{\text{firmness of loaf made with lard}}$.

[2] No bread made.

These tests indicate that alcohol-insoluble (but water-soluble) starch, (chiefly amylose) when substituted for part of the flour, increased the firmness of the loaf of bread made with monoglyceride shortening, but had little effect on the loaf made with lard. This is explainable on the basis that in the loaves made with lard, water soluble starch (amylose) was released from the starch granules, and the substitution of water soluble starch (amylose) for 5% of the flour did not materially increase the total released water-soluble starch. Although inconclusive, these tests indicate that the firmness of bread is increased by the addition of this soluble starch to the dough. In bread made without monoglyceride shortening the firmness of the bread may be increased by increased release of amylose from the starch granules. When monoglyceride shortenings are used in bread, the release of water soluble starch is retarded, and consequently a more tender and softer loaf is obtained.

Discussion

These experiments have demonstrated that monoglycerides inhibit or delay the swelling of wheat starch and possibly appreciably inhibit the release of the water-soluble starch. These results verify a prediction made by Schoch and French (16) who stated that monoglycerides would reduce the swelling power of starch, the amount of water-solubles and influence the iodine-color reaction. This effect is more pronounced in dilute flour or starch dispersions. In more concentrated systems such as bread, the effect is less, resulting in smaller quantities of water-soluble starch. Schoch and French (16) believe that this decrease of water-soluble amylose in bread is due to the precipitation of the amylose fraction during baking.

Results of the iodine tests, although not conclusive would indicate that some soluble starch, possibly amylose, was released in the breads made without monoglycerides, while the presence of monoglyceride inhibited the release of soluble starch. The exact reaction producing this effect is not known but might possibly result from the monoglycerides reacting similarly to fatty acids which Schoch (15) has shown to be adsorbed by the linear component of starch (i.e., the amylose fraction). Lord (12) has shown that polyoxyethylene monostearate depresses the swelling of starch and has a "shortening action" which softens bread crumb.

Various chemicals are known to affect the gelatinization of starch, some substances retarding and others accelerating the process (11, 13). Polyoxyethylene stearate (3) retards the gelatinization of starch but has no effect on gluten. Amylodextrin (5) lowers the gel strength and swelling power of starch. Compounds with two hydrophilic groups (such as the monoglycerides) are known to reduce the swelling of starch more than those compounds containing only one hydrophilic group (9). The swelling of starch was increased by removing some of the natural fat of the starch granule by Soxhlet extraction of the starch with methanol (6). The evidence indicates that some fats, fatty acids, fatty acid derivatives, and many other chemicals can retard or inhibit the gelatinization of starch.

The softness of bread is definitely affected by the same agents which affect starch gelatinization. Many authorities (4, 16) believe that the problem of staling in bread is associated with changes within the starch granule. Many substances which will affect the firmness and firmness changes in bread will also affect starch swelling and retrogradation. Various substances including acetaldehyde will inhibit the swelling of starch and will also prevent the staling of bread by retarding the retrogradation of starch (8).

Small quantities of monoglycerides in shortening have a definite softening effect on bread and a retarding effect on staling of bread as measured by firmness readings made with the Bloom Gelometer (7). These effects are produced by the monoglycerides acting on the starch fraction of bread.

A possible hypothesis explaining the action on monoglycerides in bread tenderizing and staling can be postulated. Microscopic examination of dough made with colored fat has shown that the large monoglyceride masses are subdivided during mixing into small globules which are dispersed between the starch granules. Some of these monoglycerides chemically attach to the starch fractions forming a loose complex while others mechanically cover parts of the starch granules, and thus reduce the capacity of the starch granules to absorb water. This delays or inhibits the swelling of the starch granule and its subsequent collapse or rupture. During baking the starch granule swells but not as rapidly nor as much as if the monoglyceride were absent. The decreased moisture absorption of the starch granule makes more moisture available for the hydration of the gluten. The decreased moisture content of the starch granule and the increased moisture content of the gluten could partially account for prolonging the softness of bread and for the slower staling rate, since the addition of fully-hydrated gluten to dough during the making of bread resulted in a loaf which kept fresh for a longer period of time (10).

Another factor to be considered in this hypothesis is the effect of the amount and distribution of the soluble starch fraction on the plasticity of the air cell wall. Air cell walls of bread are composed of starch granules and gluten strands both of which are held together with some cementing substance, possibly the "soluble starch." A decrease in the amount of released soluble starch results in smaller amounts deposited on the gluten strands or between the starch granules. When the soluble starch is released from the starch granules it forms a continuous film of this cementing substance between the starch granules and gluten strands, and the rigidity of the air cell walls and the firmness of the bread is increased. On the other hand, if the soluble starch is retained within the granules in discontinuous aggregates, the rigidity of the air cell walls is decreased and the firmness of the bread is decreased. It is likely that monoglyceride shortenings produce softer bread by (1) keeping the soluble starch within the starch granule and (2) by decreasing the gelatinization of the starch thereby allowing an increase in the moisture available for the hydration of the gluten.

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THE INFLUENCE OF VARIOUS SUGARS ON DOUGH AND BREAD PROPERTIES [1]

HAROLD N. BARHAM, JR.,[2] AND JOHN A. JOHNSON [3]

ABSTRACT

The influence of sucrose, glucose, fructose, and invert sugar on bread and dough properties was studied with the sponge dough process.

The four different types of sugars were fermented essentially at the same rate in bread doughs. Specific loaf volume increased up to 3% and decreased markedly above 6% sugar concentration. Proof times were minimum with approximately 3% sugar. Specific volumes were directly related to gas production values while proof times were indirectly related. Sugar concentrations higher than 6% caused a decrease in both the rate and the amount of gas production. Optimum grain and texture were obtained from 6 to 8% sugar concentration. Variation in specific volume among flours, in response to sugar concentration was related to susceptibility of the starch to amylase attack.

Glucose produced slightly less browning of the crust at low concentrations than the other sugars, but differences in the browning of the toast with the various sugars were not significant. Increasing sugar concentrations increased the extent of browning during baking and toasting.

Crumb firmness was affected by sugar concentration and time of storage. Minimum crumb firmness after three days of storage was observed at 2–4% concentration for all sugars. The sugars, however, did not retard firming as was shown by increased crumb firmness after three days of storage. All sugars had the same influence on firmness.

Few investigators have compared directly sucrose and glucose for bread baking purposes, although both are used extensively in commercial bread production. Furthermore, most of the published literature deals with their use in the straight-dough formula whereas most of the white bread produced commercially in the United States is made with the sponge-dough process. In the latter process, the sugar is not added until after an extended fermentation and the effects of the sugars may be different from those found for a straight-dough baking procedure. Also, much of the work has dealt with alcoholic fermentation rather than with the effects of the two sugars on bread quality.

There is some lack of agreement in the literature concerning the utilization of sucrose and glucose during bread dough fermentation and

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the effect of residual sugars on bread quality. Weiss (20, 21) has implied that glucose in bread doughs is more readily and completely fermented than sucrose. He stated that with glucose, insufficient sugar remained at the end of normal fermentation to produce a loaf of bread with a desirable brown color, a pleasant flavor, and moisture-retaining properties. Hopkins and Roberts (11) demonstrated that glucose in liquid media was fermented at a slightly greater rate than fructose at concentrations greater than 2%. At concentrations below 2%, glucose was fermented at an appreciably higher rate than fructose. Rice (17) found that yeast preferred glucose to other sugars and that by proper adjustment of the quantity of sucrose, sufficient fructose may remain to enhance the moisture-retaining quality and sweetness of the bread. Geddes and Winkler (10), however, believed that with normal concentrations of sucrose (5%) insufficient fructose remains to affect materially the moisture-retaining properties. They also showed that the rate of inversion of sucrose is very rapid and does not limit the rate of fermentation. The data of Larmour and Bergsteinsson (15) on gas production does not appear to support the conclusions of Weiss (20, 21) since sucrose, fructose, and glucose were fermented in dough essentially to the same extent.

The effect of the nature and quantity of sugars used in bread-making may be influenced by several factors (2). Gas production with the straight-dough formula has been related to naturally occurring sugars (5), amylase content (6, 8), yeast nutrients other than sugar (15), to the availability of starch to amylase attack (8), to yeast concentration (19), and temperature (18). Variations might therefore be expected in the length of time that a dough will maintain an active fermentation (14, 16, 4) with different sugar concentrations. Thus, Lanning (14) reported that 5% sucrose, 5% maltose, and sugarless doughs gave the same gas production until the four-hour period at which time little additional gas was formed in the sugarless doughs. However, the data of Larmour and Brockington (16) and Blish and Hughes (4) do not agree with those of Lanning (14). Blish and Hughes noted that 2.5% and 10% sucrose maintained an active fermentation for four and 10 hours, respectively. They did not observe that higher concentrations of sugar in doughs caused inhibitory effects on fermentation.

It is generally recognized that sugars have only a slight effect upon the retention of crumb softness during storage (3, 9, 10, 12). Edelmann, Cathcart, and Berquist (9) noted that increasing the sucrose in the formula from 4 to 12% had little effect on the rate of firming of the crumb.

Since few data are available which compare directly the effects

of glucose, fructose, invert sugar and sucrose with a commercial sponge-dough process, it was the object of this study to evaluate the effect of these sugars in varying concentrations on dough and bread characteristics.

Materials and Methods

While several flours were used during the course of this study, only four flours were chosen for the purpose of this presentation. Two were commercially milled from hard red spring wheat and two from hard red winter wheat. The analytical characteristics of these flours are shown in Table I. Moisture, ash, and protein were determined in the customary manner. The Brabender Farinograph was used to determine mixing characteristics. The amylograph viscosities were determined using 65 g. of flour (14% moisture basis) and 470 ml. of distilled water.

TABLE I

SUMMARY OF FLOUR CHARACTERISTICS

Flour Type	Protein[1]	Ash[1]	Mixing Time[2]	Absorption[2]	Amylograph Viscosity[3]
	%	%	*min.*	%	*B.U.*
Spring wheat flours					
High protein	13.3	0.47	7.0	68	205
Patent	12.2	0.41	6.0	67	170
Winter wheat flours					
High protein	12.9	0.44	7.5	69	180
Patent	11.7	0.45	6.0	69	190

[1] 14% moisture basis.
[2] Determined from the farinogram. Expressed as percent of water required to give a maximum consistency of 500 B.U.
[3] Determined on flour as received from mill. Values represent peak viscosity obtained with 65 g. flour (14% moisture basis) and 470 ml. of distilled water.

Maltose values, reducing and non-reducing sugar content were measured by A.A.C.C. methods (1).

Four sugars, glucose,[4] sucrose, fructose, and invert sugar were used. The invert sugar consisted of equal parts of glucose and fructose.

The sponge baking procedure as reported by Johnson and Miller (13) was followed. The formula is given in Table II. The method involved fermentation of a 70% sponge for four hours at 80°F. and 90% relative humidity. Depending on the nature of the experiment, either a Hobart (A200) laboratory mixer or a horizontal Day mixer was used. Optimum mixing times were employed. The floor time was usually 30 minutes. The doughs were scaled to 18 oz. by hand or by machine. They were proofed at 95°F. and 95% relative humidity for the required time which depended on the nature of the experi-

[4] Glucose used in this investigation was a commercial grade dextrose supplied by Corn Products Sales Co., Inc.

ment. The bread was baked for 30 minutes at 425°F. Loaf volumes were determined by seed displacement but expressed as specific volume, the volume in ml. of 100 g. of bread. Grain and texture scores were determined by visual examination approximately 24 hours after baking.

In experiments which involved proofing to constant height, doughs with sugar concentrations from 1 to 10% were proofed approximately 1.5 cm. above the pan edge. Sugarless doughs were proofed for 70 minutes but never reached the stipulated height.

Firmness of bread crumb was determined with a Bloom Gelometer essentially as reported by Conn, Johnson, and Miller (7).

TABLE II

SPONGE DOUGH FORMULA

Ingredient	*Sponge Dough*	
	Sponge	*Dough*
	%	%
Flour	70.0	30.0
Yeast	2.0	—
Water	Variable	Variable
Yeast food (Arkady)	0.5	—
Malted wheat flour	0.5	—
Sugar (sucrose, glucose or fructose)	—	0 to 10
Dried milk solids	—	2.0
Paniplus	—	0.5
Salt	—	2.0
Shortening	—	3.0

Tests of toasting quality of bread were made with a conventional continual band toaster of 18-slice capacity. The toaster was given a 10-minute pre-heating period before starting the tests.

Gas production and retention measurements were made by a modified A.A.C.C. method (1). Eight-gram pieces of the doughs, as prepared for baking, were placed in the Sandstedt-Blish pressuremeter cups over 23% solutions of sodium chloride and sodium hydroxide for measurement of gas production and retention, respectively. A paraffin coating was used to protect the cups from the strong alkali.

Results and Discussion

Comparison of Glucose, Fructose, Invert Sugar, and Sucrose in Baking. The first experiments were concerned with demonstrating the effect of sucrose and its components, glucose and fructose, on the quality of bread. Results for a typical flour (high protein, hard red winter) are shown in Figs. 1, 2, and 3 and in Tables III and IV. The specific volume of the loaves (Fig. 1) increased sharply up to 2%

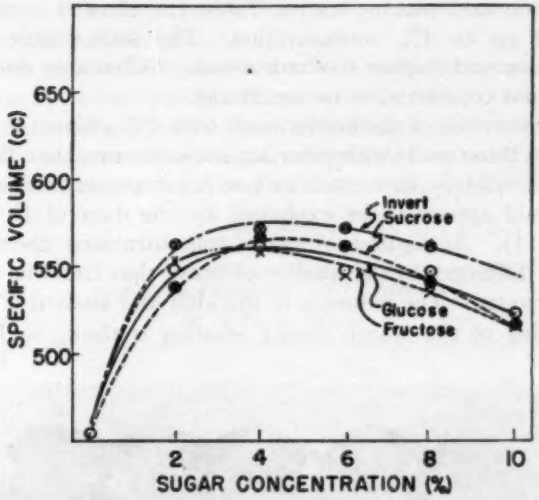

FIG. 1. The effect of sugar type and concentration on specific loaf volume.

concentration for all sugars. The change from 2 to 6% was generally small and as the concentration was increased from 6 to 10% the specific volume decreased steadily. Although no data are presented, essentially the same trends were observed with the straight dough procedure. The decrease in loaf volume when high concentrations of sugar are used is commonly recognized in commercial practice. It is customary to increase the percentage of yeast when high sugar concentrations are employed to overcome any reduction of loaf volume. Later experiments revealed that higher sugar concentration reduced both the rate and the amount of gas production in a fermenting dough.

TABLE III

GRAIN AND TEXTURE SCORES [1] FOR BREAD BAKED WITH VARIOUS CONCENTRATIONS OF GLUCOSE, SUCROSE, FRUCTOSE, AND INVERT SUGARS

Sugar Concentration	Glucose		Sucrose		Fructose		Invert	
	Grain	Texture	Grain	Texture	Grain	Texture	Grain	Texture
%								
0	70	65	70	65	70	65	70	65
2	80	80	65	80	82	80	80	75
4	85	83	80	85	82	82	82	85
6	85	90	88	88	85	85	85	92
8	90	90	88	85	85	88	88	92
10	86	90	86	86	85	88	85	90

[1] Grain and texture scores were relative and based on 100 maximum score.

The grain and texture scores, Table III, showed a marked improvement up to 4% concentration. The scores were improved slightly more with higher concentrations. Differences due to sugar type were not considered to be significant.

The crust colors of the loaves made with 2% glucose were slightly lighter than those made with other sugars, indicating that glucose may be more completely fermented at low sugar concentrations. These results would appear to be explained by the data of Hopkins and Roberts (11). At optimum sugar concentrations there was no significant difference in the quality of bread that could be attributed to kind of sugar. The influence of the kind and amount of sugars on the browning of the crumb during toasting is shown in Fig. 2. If

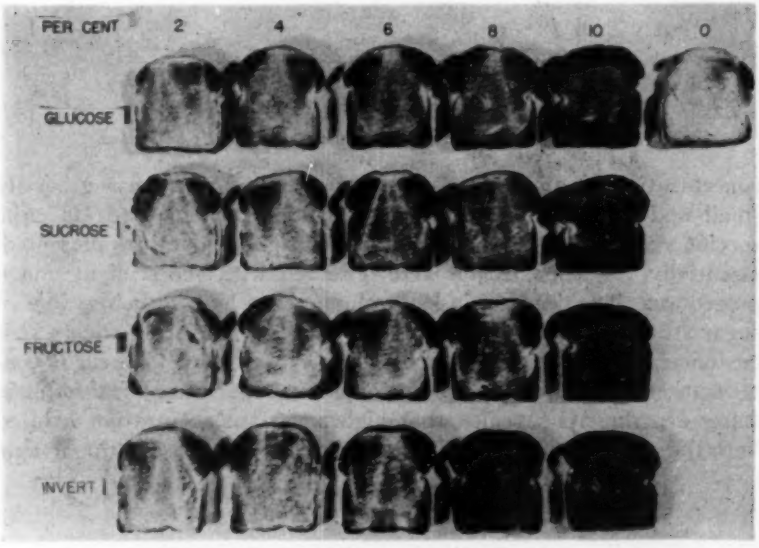

FIG. 2. The effect of sugar type and concentration on toasting quality of bread.

glucose were more readily and completely fermented than sucrose or fructose, a difference in browning of the toast might be expected. Such differences were not apparent. Thus, there appears to be no practical difference in the rate of utilization of the various sugars. Increasing sugar concentrations, however, caused more pronounced browning.

Measurement of the crumb firmness (Table IV) showed that firmness of bread crumb was significantly affected by both sugar concentration and days of storage. All sugars influenced crumb firm-

ness in a similar manner as shown by the non-significant effect of sugar type (Table IVb).

The effect of sugar concentration on crumb firmness at the end of three days of storage was significant and very apparent to the touch. Minimum crumb firmness was observed from 2 to 4% sugar concentration. As the sugar concentration was increased beyond 4% the crumb became increasingly firm. The firmness of the crumb with sugar

TABLE IV

EFFECT OF CONCENTRATION OF VARIOUS SUGARS ON CHANGE IN CRUMB FIRMNESS[1] WITH STORAGE

A. Table of Means

Sugar Used	Day	Concentration					Mean
		2	4	6	8	10	
Glucose	1	68	64	69	66	75	68
	3	104	102	108	107	118	106
Sucrose	1	68	68	68	80	88	74
	3	107	96	104	107	111	105
Fructose	1	76	74	78	74	90	78
	3	128	100	120	102	117	114
Invert	1	77	79	62	69	89	75
	3	106	104	107	115	122	110
Mean		92	86	90	90	101	91

B. Analysis of Variance

Source of Variation	Degrees of Freedom	Mean Squares
Sugar[2]	3	843
Concentration[2]	4	1,615[4]
Day[2]	1	72,350[4]
Sugar × day[2]	3	121
Sugar × concentration[2]	12	279
Concentration × day[2]	4	235
Sugar × day × concentration[3]	12	247[4]
Error	200	27
Totals	239	

[1] Crumb firmness determined by Bloom gelometer represents grams of lead shot required to depress a 1-in. diameter plunger into a uniform slice of bread to a depth of 4 mm.
[2] Significance tested by sugar × day × concentration interaction term.
[3] Significance tested by residual error.
[4] Significant to the 1% level.

concentrations higher than 4% while related to loaf volume, was more firm than could be accounted for by volume differences. It may be that increasing amounts of sugar served as a bonding force and therefore created a firmer and less resilient crumb.

The effect of varying glucose and sucrose concentration on proof time is shown in Fig. 3. While there was no significant difference in the effect of glucose and sucrose on the proof time, sugar concentrations

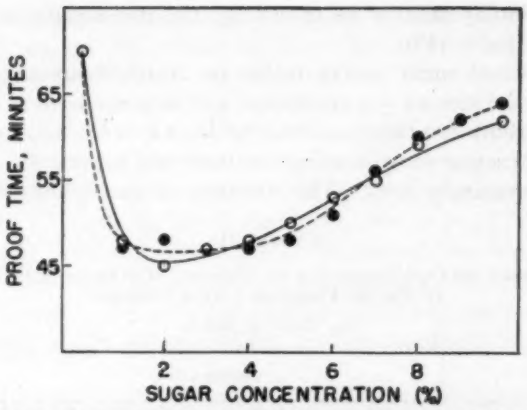

FIG. 3. The effect of sugar type and concentration on proofing time. Solid line represents glucose and the broken line represents sucrose.

higher than 4% materially increased proofing time. This suggested that high sugar concentrations inhibited fermentation. Other experiments established that high sugar concentration reduced the rate of gas production. This also explained the effect of sugar concentration on specific volume observed in Fig. 1.

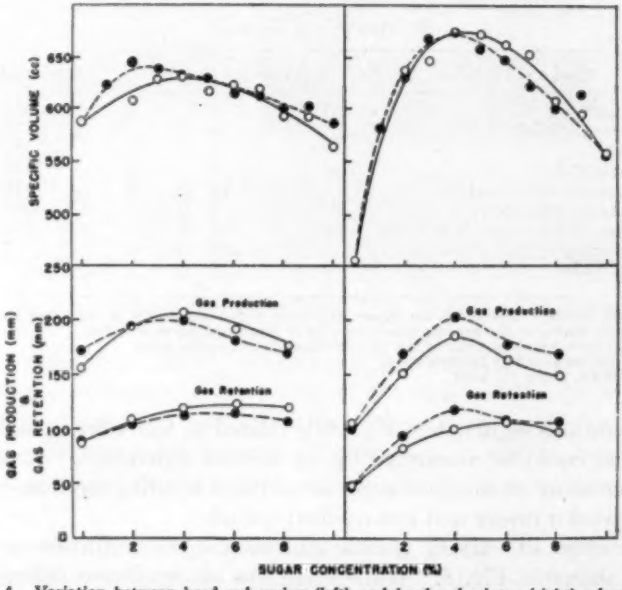

FIG. 4. Variation between hard red spring (left) and hard red winter (right) wheat flour in response to sugar concentration. The solid line represents glucose and the broken line represents sucrose.

Response of Flours to Different Sugar Concentrations. The response of different flours to changes in the sugar concentration varied. The effects of glucose and sucrose concentration on specific volume, total gas production and gas retention for two flours exhibiting different response are shown in Fig. 4. The winter wheat flour exhibited a more marked response in specific volume and total gas production than the hard red spring wheat flour. An adequate rate of gas

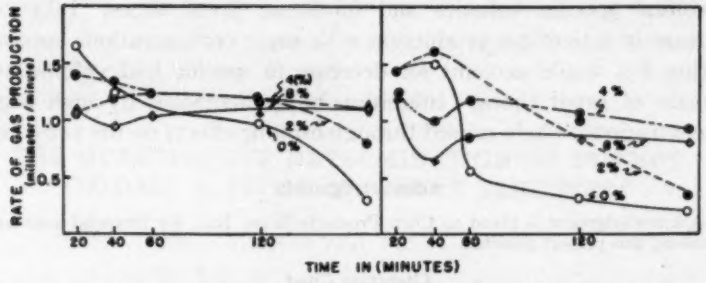

FIG. 5. Effect of glucose concentration on rate of gas production. Hard red spring on left and hard red winter wheat flour on right.

production for sugarless doughs was maintained slightly more than 40 minutes with the one flour whereas, with the other, a sharp decrease in rate did not occur until after 120 minutes as is shown in Fig. 5. Analysis for reducing and non-reducing sugars indicated that the two flours were not greatly different in total natural sugars, as shown by the following flour-sugar analysis.[5] Likewise, the maltose values were

Flour	Sugar Content		Maltose Value	
	Red.	Non-red.	Original	Diastated
	mg./10 g.	*mg./10 g.*	*mg./10 g.*	*mg./10 g.*
Spring wheat patent	9.0	19.0	329	546
Winter wheat patent	5.5	19.0	327	421

approximately equal. However, with the addition of 1% of malted wheat flour in the formula, the maltose value of the hard red spring wheat patent was more than 100 mg. greater than the corresponding hard red winter. Gas retention was slightly improved with optimum sugar concentration of 4 to 6% and could not account for the variation in specific volume response of the different flours to increasing sugar concentrations. Thus, it appears that the variation among flours in

[5] Expressed on a 14% moisture basis.

response to sugars as noted in specific volume may be associated with the availability of the starch to amylase attack.

The effect of increasing concentration of sugars on rate of gas production is shown in Fig. 5. While increasing concentrations of sugar above 4% caused the rate of gas production to become more constant with extended fermentation time, the rate was lowered. With sugar concentrations from 2 to 4% in the sponge dough, the rate of gas production was sufficiently large and steady to account for the maximum specific volumes and minimum proof times. Likewise, decrease in rate of gas production with sugar concentrations approximating 8% would account for decrease in specific loaf volume and increase of proof times. Inhibition of fermentation by high sugar concentration is likely caused through osmotic effects on the yeast cell.

Acknowledgments

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THE QUANTITATIVE DETERMINATION OF PHENOL OXIDASE ACTIVITY IN WHEAT VARIETIES [1]

MAX MILNER and M. R. GOULD

ABSTRACT

A method for the determination of catechol oxidase activity in dry plant tissues which involves titration with 2,6-dichlorophenolindophenol of ascorbic acid remaining after partial destruction of this compound by the enzymic oxidation product of catechol, was used in a preliminary study of the phenol oxidase activity in the entire wheat and bran of ten pure varieties of hard red winter and four of hard red spring wheats. Marked variation in catechol oxidase activity among different wheat varieties was found, and the activity of the bran was roughly four times that of the entire wheat in all cases. The oxidase activity of wheat bran on several phenolic substrates showed major differences at similar concentrations of the phenol, the extent of activity increasing with the number of functional hydroxyl groups in the compound.

The brans from different wheat varieties show similar differences in oxidase activity when catechol, pyrogallol and *p*-cresol are used as substrates. However, when the ratios of the activities of each bran acting on the three substrates were calculated, it was found that the relative activity on pyrogallol decreased in the same order in which the varieties were shown to increase in activity when acting on catechol. The possible utility of such methods for biochemical differentiation of wheat varieties is discussed.

Wheat bran is known to contain an enzyme in considerable concentration capable of oxidizing phenol and related compounds although little is known concerning its other properties. A number of workers have employed a so-called "phenol color reaction" using intact wheat kernels with the objective of differentiating wheat classes and varieties (2, 4, 5, 9). This test, which is carried out simply by soaking the

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Contribution No. 185, Department of Milling Industry, Kansas Agr. Expt. Station, Manhattan, Kansas.

grain in dilute phenol solutions and then judging the color changes which occur, appears to be highly empirical and difficult of precise interpretation.

The scarcity of information regarding the properties and distribution of this enzyme in wheat doubtless has been due in part to the lack of suitable quantitative methods for the determination of the enzyme in dry tissues, although several excellent procedures for the determination of phenol oxidase activity in aqueous extracts are available (1, 3, 6, 8). Since the initiation of the present studies, Wallerstein *et al.* (11) have published a method for the determination of pyrogallol oxidase activity in cereals which employs homogenates as sources of enzyme and in which the activity is determined colorimetrically from the concentration of a colored oxidation product formed during the reaction which is extractable from the reaction mixture with ethyl acetate.

The method recently developed by one of the authors, which has been used to determine the relative activity of catechol oxidase in a number of cereal grains as well as flaxseed and soybeans (7) is well adapted to an investigation of phenol oxidase activity in dry wheat tissues. In the present study the relationship of variety to enzyme activity in ten pure varieties of hard red winter wheat and four varieties of hard red spring wheat was determined. In addition, a preliminary study of the differential action of varieties on several phenolic substrates was carried out.

Materials and Methods

The titration method for catechol oxidase activity developed by Milner (7) was used. In principle, the method involves the oxidation by benzoquinone, formed from catechol by enzyme action, of ascorbic acid present in excess in the reaction mixture (see p. 436, *this Journal*, for equations). The extent of enzyme activity is then determined by quantitative estimation of the residual ascorbic acid in the mixture. In this procedure, a weighed quantity of finely ground dry plant tissue (0.5 g. of bran and 1.0 g. of wheat were found suitable) is incubated at 25°C. in a reaction mixture containing 25 ml. of citrate-phosphate buffer, pH 5.2, 5 ml. of water, 10 ml. of 1% ascorbic acid solution and 10 ml. of 1% catechol solution. The reaction is carried out for 1 hour in 125 ml. Erlenmeyer flasks which are agitated in a 25°C. thermostat by a shaking mechanism while oxygen is passed continuously through the flasks. At the end of the hour, 50 ml. of a mixture of metaphosphoric and acetic acids are added to stop the reaction and after filtering, a 10 ml. portion is titrated for residual ascorbic acid, with 2,6-dichlorophenolindophenol. Blank values were determined and deducted from the over-all activity determinations inasmuch as some

destruction of ascorbic acid occurs due to oxygen alone as well as to the catalytic effects of materials in the wheat which remain after the phenol oxidase enzyme has been inactivated by boiling. Correction for ascorbic acid oxidase activity was shown to be unnecessary in preliminary tests because only insignificant amounts were found to occur in wheat. Oxidase activity is generally expressed in terms of milligrams ascorbic acid destroyed by the action of 1 g. of enzyme-containing material in one hour.

For the study of the relationship of wheat varieties to enzyme activity ten samples of pure variety hard red winter wheat composites and four composites of hard red spring wheat varieties were used. The winter wheat variety composites were a mixture of equal parts of pure variety samples grown at six stations located in various sections of Kansas, while the hard red spring wheat varieties were composited from samples grown at two stations in the United States spring wheat area. A portion of each composite was tempered, and milled with the Buhler mill to an average extraction of 68% and the bran was retained. Samples were milled in sequence but collection of the bran from the mill was started only after one-third of the sample had been milled, to insure that no contamination of one sample by another would occur. For enzyme activity studies, portions of the original wheat as well as the bran were ground in the intermediate Wiley mill to pass the No. 40 sieve.

Preliminary to studies of phenol oxidase activity on different substrates, the influence of substrate concentration on activity was carried out using the following compounds: gallic acid, phenol, *p*-cresol, catechol and pyrogallol. These were selected for study from a larger group of materials because of their satisfactory water solubility. Of these, catechol, *p*-cresol, and pyrogallol were selected for study with brans milled from a number of the pure wheat varieties.

Results and Discussion

Catechol Oxidase Activity of Hard Red Winter and Hard Red Spring Wheat Varieties. The oxidase activity of entire wheat and bran of ten hard red winter wheat varieties and four hard red spring wheat varieties using catechol as a substrate is shown in Table I. These data indicate that significant differences in activity exist among wheat varieties and it is probable, therefore, that phenol oxidase activity in wheat is governed by genetic factors. It is of interest that the activity of the whole wheat is approximately one quarter that of the bran for all of the 14 varieties listed. Since the ratio of bran to other products obtained in milling is also approximately 1:4, these results suggest that virtually all the phenol oxidase activity

occurs in the bran fraction. This was shown to be true in related studies not yet published.

Influence of Phenolic Substrate. Results of the study using various phenols as substrates with wheat bran are given in Table II. Gallic acid was the only one which appeared to inhibit the enzyme as indicated by a decrease in activity with increasing concentration of the compound. Other substrates including phenol, *p*-cresol, catechol and pyrogallol showed increasing activity as substrates in the order indicated. It is of interest that this increase in activity is in the same order as the increase in the number of functional hydroxyl groups in the polyhydric phenols.

TABLE I

CATECHOL OXIDASE ACTIVITY OF WHEAT VARIETIES

Variety	Catechol Oxidase Activity	
	Wheat	Bran
Hard Red Winter Wheats		
	(*mg. ascorbic acid per g./hr.*)	
Blue Jacket	6.1	27.0
Triumph	8.7	35.2
Westar	14.6	59.8
Pawnee	22.0	89.1
Turkey	26.6	104.2
Wichita	32.6	107.7
C.O.T.	26.7	108.0
Kawvale-Marquillo × Kawvale-Tenmarq	31.1	110.1
Comanche	29.4	110.2
Red Chief	28.9	116.8
Hard Red Spring Wheats		
Mida	18.2	71.8
1831	19.7	75.2
Thatcher	22.7	97.9
Rushmore	26.7	101.0

Relative Activity of Wheat Varieties on Different Substrates. With the objective of determining whether the bran of different wheat varieties might exhibit differential activity on various substrates, the oxidase activity of the bran of five hard red winter wheat varieties and four spring wheat varieties were determined using catechol, *p*-cresol and pyrogallol as substrates. Results of this study appear in Table III. The varieties are listed in increasing order of activity on catechol and the corresponding activities with *p*-cresol and pyrogallol substrates appear in adjoining columns. In general, the activity with these substrates paralleled the activity on catechol, although minor inversions occur as in the case of brans from the varieties Red Chief and Rushmore acting on pyrogallol.

TABLE II

INFLUENCE OF VARIOUS CONCENTRATIONS OF SUBSTRATE IN THE REACTION MIXTURE ON PHENOL OXIDASE ACTIVITY OF WHEAT BRAN

Phenolic Substrate	Concentration	Phenol Oxidase Activity
	mg.	*mg. ascorbic acid/g./hr.*
Gallic acid	50	8.0
	100	4.7
	150	4.1
Phenol	50	15.0
	100	17.6
	150	22.3
p-Cresol	50	60.9
	100	71.6
	150	75.2
Catechol	100	108.8
Pyrogallol	50	83.9
	100	100.2
	200	110.4
	300	112.5

When the catechol oxidase activity is held as unity and the relative activity on the other substrates is calculated, the ratios listed in the last column of Table III are obtained. Activity on *p*-cresol showed no definite trend in the case of hard red winter wheat varieties but remained almost constant for the hard red spring wheat varieties.

TABLE III

PHENOL OXIDASE ACTIVITY OF BRAN OF DIFFERENT WHEAT VARIETIES ON VARIOUS SUBSTRATES

Variety	Substrates			Activity Ratio		
	Catechol	*p*-Cresol	Pyrogallol	Catechol	*p*-Cresol	Pyrogallol
Hard Red Winter Wheat						
Blue Jacket	29.5	24.3	43.0	1	0.82	1.46
Triumph	36.8	17.0	50.2	1	0.46	1.37
Pawnee	91.5	51.9	102.9	1	0.57	1.13
Comanche	109.7	77.2	123.2	1	0.70	1.12
Red Chief	114.4	85.9	112.5	1	0.75	0.98
Hard Red Spring Wheat						
Mida	72.3	43.5	97.5	1	0.60	1.35
1831	76.5	48.9	101.3	1	0.64	1.32
Thatcher	97.8	56.2	119.9	1	0.58	1.23
Rushmore	102.5	68.1	120.1	1	0.66	1.17

The relative activity on pyrogallol, however, decreased in an apparently significant manner relative to the catechol oxidase activity of these varieties, in both classes of wheat. These results suggest that the pyrogallol oxidase activity of wheat may be due to an enzyme other than that which oxidizes catechol and that significant differences in relative concentration exist between the two enzymes in a given variety. It is of interest in this connection that Wallerstein *et al.* (11) have found a pyrogallol oxidase in wheat which they believe to be a different enzyme than the one which oxidizes *o*-phenylenediamine which they had previously noted in wheat (10).

The titration method for catechol oxidase in dry plant tissues appears to offer a useful tool for the investigation of the relationships between wheat variety and the concentration or activity of enzymes which oxidize phenols. In this study, wide quantitative differences have been shown to exist in the catechol oxidase activity of hard red winter and hard red spring bread wheat varieties and it seems possible that tests of this nature may be useful for the biochemical identification of wheat varieties. Further studies along this line are in progress.

Acknowledgments

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DIRECT EFFECT OF POLLEN PARENT ON PROTEIN CONTENT OF THE CORN KERNEL [1]

EARL R. LENG,[2] F. R. EARLE,[3] and J. J. CURTIS[3]

ABSTRACT

Direct effects of pollen parent on total protein content of the corn kernel were studied by pollinating silks of Illinois High Protein and Illinois Low Protein corn with two mixtures of pollen from white and yellow types of corn differing in protein content. Kernels produced on the same ear but with different pollen parents were separated by xenia effects on endosperm color.

Statistically significant differences in protein content attributable to pollen source were found in three of the four 50-ear sets studied, the greatest mean difference being 0.36%. Although statistically significant, the differences in protein content resulting from direct effects of pollen were so small that they are considered to be of no practical importance. Since it has previously been shown that incomplete seed-setting on the corn ear can produce much greater changes in protein content of the grain than those found to result from the direct effects of different pollen parents, it is suggested that the protein-producing capacity of a given genotype in corn can best be measured by allowing open-pollination in order to insure a full seed set.

Breeding for high protein content of the kernel has become an important objective of several corn improvement programs. It has been shown that protein content of corn can be greatly altered by mass selection (9), and the feasibility of developing high-protein hybrids by selection during inbreeding has long been established (5). Knowledge of the mode of inheritance of protein content is important in planning breeding programs, and has been the object of several investigations (1, 2, 3, 6, 7).

Information on effects of the pollen parent on protein content of the kernel is necessary before the accurate representation of the protein-producing capacity of a given genotype can be assured. Hayes and Garber (5) indicated that effects of the pollen parent were not important, in comparing grain from selfed and crossed ears of the same inbred lines. Tests of pollen effects reported by these workers were not critical, since differences between selfed and crossed kernels

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[3] Chemist and Agricultural Technologist, respectively, Analytical and Physical Chemical Division, Northern Regional Research Laboratory.

which were found were attributed to poor seed set on the crossed ears. Results of reciprocal crosses between Illinois High Protein and Illinois Low Protein also indicated that effects of the pollen parent on protein content were not great (8). Frey (2) pointed out that his material was "selfed to eliminate any effects of foreign pollen" and also suggested that low protein content was completely dominant to high protein content. Similar dominance relationships had been reported by early workers (3, 7).

Reported complete dominance of low protein over high protein content suggested the possibility that important effects of the pollen parent on protein content might occur if pollen from low-protein strains fertilized silks of high-protein strains. The experiments reported in this paper were conducted to provide a critical test of this possibility, and to determine if pollen source had any measurable effect on protein content of the corn kernel.

Materials and Methods

Illinois High Protein and Illinois Low Protein strains of corn were used as ear parents in the studies reported in this paper. Selection for high and low protein content, respectively, was begun in these strains in 1896 and had been in progress for 49 generations at the time seed was selected for use in these tests. Silks of 50 ears of each strain were pollinated with each of the following two-pollen mixtures:

(1) Illinois High Protein (white) and inbred Oh28 (yellow), and
(2) Illinois Low Protein (white) and inbred 48–234 (yellow).

Oh28 was selected as a pollen parent because it had been found [4] to contribute low protein content to its hybrids. Inbred 48–234 was derived from a back-crossing program involving Illinois High Protein.[5]

Kernels produced on the same ear but with different pollen parents were separated by using color differences resulting from xenia effects. Adjacent yellow and white kernels were taken in pairs, 25 kernels with each pollen parent constituting the pair of samples taken from each ear. There were thus 50 pairs of samples of each strain, each pair being taken from a different ear.

The samples were analyzed for total nitrogen content by the A.O.A.C. Kjeldahl-Gunning-Arnold method with the exception that boric acid was used to receive the distilled ammonia. Since the samples were artificially dried to uniform moisture content after harvest, analyses are reported on an air-dry basis. Total protein percentage was found by multiplying total nitrogen percentage by 6.25.

[4] Unpublished data.
[5] The authors are indebted to Dr. R. W. Jugenheimer for furnishing seed of these inbred lines.

Results and Discussion

Statistically significant differences in total protein content attributable to pollen source were found in three of the four 50-ear sets studied (Table I). When Illinois High Protein was used as the ear parent, its own pollen produced kernels averaging 0.36% higher in protein than kernels with Oh28 as male parent. High Protein pollen also produced kernels significantly higher in protein content than did pollen of Oh28, when Illinois Low Protein was the female parent.

TABLE I

EFFECT OF POLLEN SOURCE ON TOTAL PROTEIN CONTENT OF HIGH PROTEIN AND LOW PROTEIN STRAINS OF CORN, 1949[1]

Pollen-Mix Male Parent	*Ear Parents*	
	Illinois High Protein	*Illinois Low Protein*
	% protein	*% protein*
Low Protein	17.69	5.45
48–234	17.45	5.44
Difference	0.24[2]	0.01
High Protein	17.43	4.86
Oh28	17.07	4.76
Difference	0.36[2]	0.10[2]

[1] Data are means of 50 individual ear samples.
[2] Exceeds the 1% level of significance (paired-method "t" test).

Sib-pollinated seed of Illinois Low Protein had less than half as great a percentage of total protein as seed of inbred 48–234 (Table II). Yet, pollen of Low Protein produced kernels significantly higher in protein than did pollen of 48–234, when High Protein was the ear parent. Low Protein and 48–234 produced kernels almost identical in protein content when used as pollen parents on ears of Low Protein. With present information, these somewhat unexpected results cannot be adequately explained.

TABLE II

PROTEIN CONTENT OF SIB-POLLINATED SEED OF TWO INBRED LINES AND TWO SELECTED STRAINS OF CORN, 1949[1]

Inbred or Strain	*Total Protein*
	%
Illinois High Protein	18.20
Oh28	9.66
48–234	9.49
Illinois Low Protein	4.60

[1] Data are means of 10 individual ear samples.

Although statistically significant, the differences in protein content resulting from direct effects of pollen were so small as to appear to be of no practical significance.[6] The greatest proportional differences due to pollen source were those between High Protein and Oh28 as pollen parents, and these differences were only about 2% of the total protein content of the ear parents. Much larger proportional differences have been shown to result from differences in seed set (4). Since accurate measurement of the protein-producing capacity of a given genotype is necessary in studies of the inheritance of protein content, it appears essential to insure complete fertilization and a full seed set to give the best possible measure of this capacity. Under normal Corn Belt conditions, this can best be achieved by using the tested strain as female parent, growing it on a highly fertile soil, allowing open-pollination to occur, and disregarding the relatively minor direct effects of pollen source on protein content. Control of pollination would appear to be necessary only when it is desired to obtain seed of known male parentage.

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[6] After the manuscript of this paper had been prepared, the authors' attention was called to an unpublished paper, "Protein Studies in Corn," read by Lawrence M. Rohrbaugh at the March, 1946 meeting of the American Society of Plant Physiologists. Methods similar to those reported in this paper were used by Dr. Rohrbaugh, who found no significant direct effects of pollen on protein content of the corn kernel.

NON-ENZYMATIC CLEAVAGE OF *p*-NITROPHENYL ESTERS [1]

B. M. Dirks and P. D. Boyer [2]

ABSTRACT

The use of acyl esters of *p*-nitrophenol, which have been suggested as substrates for estimating esterase activity colorimetrically, has been evaluated. The use of these substrates has been found unsatisfactory because of the occurrence of non-enzymatic cleavage of the esters. This cleavage is brought about by the reducing agents, glutathione, cysteine, hydroxylamine and sodium hydrosulfite. The reaction with hydroxylamine is stoichiometric and gives free *p*-nitrophenol and an alkyl aldehyde as reaction products. Native crystalline bovine serum albumin and heated crystalline egg albumin also cause the cleavage of these esters. *p*-Nitrophenyl phosphate is not split under comparable conditions.

In connection with studies on cereal and mold esterases, a procedure suggested by Huggins and Lapides (4) seemed attractive as a possible simple method for the measurement of enzyme activity. This procedure is based on the splitting of colorless acyl esters of *p*-nitrophenol to give yellow free *p*-nitrophenol, the concentration of which may be conveniently determined colorimetrically. Preliminary experiments with the procedure suggested, however, that non-enzymatic cleavage of the esters may occur under the assay conditions used. The use of the acyl esters was thus further investigated, including qualitative study of the reaction with reducing agents.

Materials and Methods

p-Nitrophenyl Esters. The acetate and propionate esters of *p*-nitrophenol (PNP) were prepared as described by Huggins and Lapides (4). The melting points of the esters used checked with those previously reported (4).

Measurement of p-Nitrophenyl Acetate (PNPA) Cleavage. Unless otherwise indicated these measurements were made in 19 mm. test tubes matched for colorimetry and containing, in a 10 ml. total volume, 0.666 micromoles PNPA, 2 ml. *M*/15 phosphate buffer, pH 7.1, and appropriate additions of reducing agents or proteins. The reaction mixture without PNPA was equilibrated in a water bath at 30°C.

[1] Manuscript received July 25, 1951. Presented at the Annual Meeting, May, 1951. Minnesota Agricultural Experiment Station Paper No. 2681, Scientific Journal Series. Supported in part by a grant from Pillsbury Mills, Inc., Minneapolis, Minnesota.

[2] Division of Agricultural Biochemistry, University of Minnesota, St. Paul 1, Minnesota. The results reported will be developed as part of a thesis to be submitted by B. M. Dirks in partial fulfillment of the requirements for the Ph.D. degree.

for 10 min. or more and then the PNPA solution, previously brought to 30°C., was added. The zero time optical density was read immediately and other readings at intervals thereafter. Optical density measurements were made using a Coleman Junior Spectrophotometer with a wave length setting of 400 mμ and compared to a standard curve for PNP. Corrections were made for blank tests made simultaneously.

Determination of Hydroxylamine. The utilization of hydroxylamine in the reaction with PNPA was measured as follows: A 2 ml. aliquot of the reaction mixture was added to 4 ml. ferric chloride solution containing 5 micromoles of ferric chloride and sufficient hydrochloric acid to give a final pH of 3.0 to 3.3. The mixture was allowed to stand for 10–15 minutes. The ferrous iron produced was measured by addition of 1 ml. of α,α'-dipyridyl solution (5 mg. per ml. in ethanol), and the resulting red color measured colorimetrically at 510 mμ. Values obtained were referred to a standard curve for hydroxylamine concentration.

Determination of Acetaldehyde. The presence of acetaldehyde in the reaction products was detected by a modification of the Barker and Summerson (1) method for determination of lactic acid. A 1 ml. aliquot of reaction mixture was added to a colorimeter tube and cooled in ice water. Six milliliters of concentrated sulfuric acid were added slowly keeping the contents of the tube cold. To this mixture was added 1 drop of 4 gram-per cent aqueous cupric sulfate and 1 drop of 1.5 gram-per cent *p*-hydroxydiphenyl in 0.5 gram-per cent sodium hydroxide. The contents were mixed and incubated for 30 minutes at 30°C. with periodic shaking. The tubes were then heated for 90 seconds in boiling water, cooled to room temperature and the optical density was measured at 570 mμ. Standard curves for acetaldehyde were prepared for reference.

Results and Discussion

Preliminary experiments showed that *p*-nitrophenyl acetate (PNPA) was actively cleaved by a wheat germ lipase preparation. A possible defect of the procedure became apparent, however, when it was noted that glutathione (GSH), added to protect the lipase, caused marked increases in the velocity of ester hydrolysis. This increase in velocity was not abolished by heating the preparation to inactivate the enzyme.

As a consequence of the observed action of GSH, tests were made with other reducing agents. Figure 1 shows the high rate of cleavage obtained with several reducing agents added to the 10 ml. of reaction mixture in the amounts indicated. Since no attempt was made to

assess the relative purity of the reducing materials available, the concentrations should be regarded as only approximate. PNPA was not split by additions of glucose, sodium thiosulfate, ferrous sulfate, glycine, or sodium dodecyl sulfate. The effect of cysteine was furthermore completely eliminated by the addition of the oxidizing agent, potassium iodate.

Certain proteins were also able to bring about the splitting of PNPA. Figure 2 shows the results of tests using crystalline bovine serum albumin, ovalbumin, and β-lactoglobulin in concentrations of approximately 10 mg. dry protein per 10 ml. of reaction mixture. The

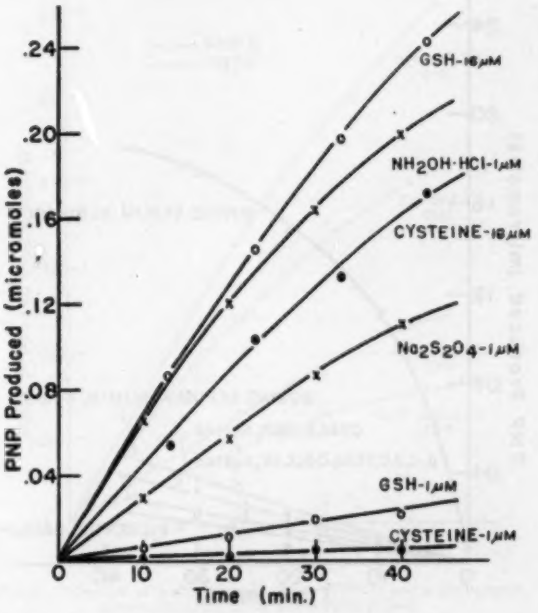

FIG. 1. Cleavage of p-nitrophenyl acetate by various reducing agents.

serum albumin is seen to be particularly active while ovalbumin showed no activity in its native state. However, after the aqueous protein solutions were heated 10 minutes in a boiling water bath, the serum albumin lost much of its activity while ovalbumin acquired significant activity. β-Lactoglobulin activity did not change materially under this treatment.

The activity of the proteins may in part be due to the presence of sulfhydryl groups. In harmony with this view, heat denaturation of egg albumin is known to increase the reactivity of its sulfhydryl

groups (7). However, other factors are probably involved, particularly in the marked effect of serum albumin. Jensen *et al.* (5) have carried out amperometric titrations which indicate that crystalline bovine plasma albumin has an average of only 0.71 sulfhydryl groups per molecule. The concentration of sulfhydryls in the albumin solution used for the PNPA reaction is then only 1/150 of that in the glutathione solution giving a comparable hydrolysis rate. Further, the total amount of sulfhydryl groups calculated on this basis is comparable to only approximately ⅓ of the amount of PNPA split

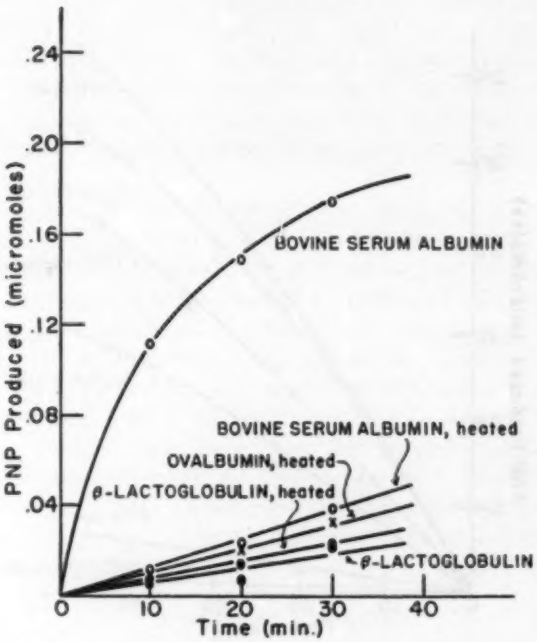

FIG. 2. Cleavage of *p*-nitrophenyl acetate by various proteins.

if two sulfhydryl groups are oxidized for each PNP molecule produced. As mentioned below, the cleavage of PNPA by hydroxylamine involves a stoichiometric reaction with the reducing agent. Whatever the mechanism of action of the proteins may be, these results indicate a possible serious error in estimation of esterase activity of biological materials with PNPA as a substrate.

Figure 3 illustrates that *p*-nitrophenyl propionate (PNPP) reacts with GSH but somewhat slower than PNPA. Esters with larger acyl groups would probably also be cleaved though at still different rates.

On the other hand, *p*-nitrophenyl phosphate, used as a substrate for alkaline phosphatase activity by Bessey, Lowry and Brock (2), was not cleaved by these reducing agents under the conditions described. This may be because the phosphate group, unlike the acyl group, is not susceptible to reduction.

Qualitative measurements showed that GSH was oxidized in its reaction with PNPA. This indicated that the splitting of the ester was not catalytic and suggested a possible "reductive cleavage" with production of free PNP and acetaldehyde. Examination of absorption

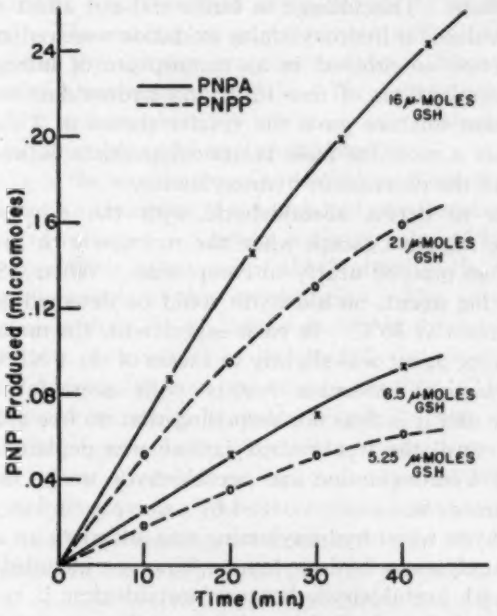

FIG. 3. Cleavage of *p*-nitrophenyl acetate (PNPA) and *p*-nitrophenyl propionate (PNPP) by glutathione (GSH).

spectra showed products of the reaction between PNPA and GSH to have the same wave length of maximum absorption as PNP. Furthermore, the optical densities of the reaction products and of PNP at 400 mμ varied similarly with changes in pH (Table I). From these results it is concluded that one of the products of the reaction is free PNP.

The use of hydroxylamine hydrochloride proved better suited than GSH for studying the quantitative relationships between PNPA and reducing agents. In order to use ferric iron in the hydroxylamine determination it was necessary first to change the phosphate buffer in the reaction mixture to a tris(hydroxymethyl)aminomethane-

TABLE I

EFFECT OF pH ON OPTICAL DENSITIES OF SOLUTIONS OF *p*-NITROPHENOL AND PRODUCTS OF CLEAVAGE REACTION

pH	Optical Density (Relative Values) PNP	Reaction Products
6.78	71	74
7.08	100	100
7.25	116	119
7.48	134	134
7.75	154	152

hydrochloric acid buffer to eliminate the precipitation of insoluble ferric phosphate. This change in buffer did not affect the reaction. High blank values for hydroxylamine oxidation were reduced by allowing the reaction to proceed in an atmosphere of nitrogen. Simultaneous determinations of free PNP and hydroxylamine in aliquots of the reaction mixture gave the results shown in Table II. It is apparent that a mole for mole relationship exists between the PNP produced and the decrease in hydroxylamine.

Attempts to detect acetaldehyde with the *p*-hydroxydiphenyl reagent were negative except when the reaction with hydroxylamine was allowed to proceed nearly to completion. When GSH was used as the reducing agent, no aldehyde could be detected even after 72 hours incubation at 30°C. In each experiment, the molar concentration of reducing agent was slightly in excess of the PNPA.

Hydroxylamine condenses readily with acetaldehyde to form acetaldoxime and it is thus not surprising that no free aldehyde could be detected until the free hydroxylamine was depleted. This condensation of hydroxylamine and acetaldehyde under the conditions of the experiment was easily verified by a decrease in intensity of tests for acetaldehyde when hydroxylamine was added to an acetaldehyde solution. Analyses for hydroxylamine, however, included that portion condensed with acetaldehyde because acetaldoxime is readily hydrolyzed under the analytical conditions. Quantitative recovery of

TABLE II

p-NITROPHENOL PRODUCED RELATIVE TO HYDROXYLAMINE DECREASE [1]

Trial No.	PNP Produced	NH_2OH Decrease	Ratio NH_2OH/PNP
	micromoles	*micromoles*	
1	0.952	1.000	1.05
2	0.894	0.940	1.05
3	0.873	0.860	0.98
4	0.823	0.960	1.17
Average			1.06

[1] Tests were made for 80 minutes at room temperature under an atmosphere of nitrogen. Twenty milliliters of test mixture included 2.5 micromoles hydroxylamine, 1.665 micromoles PNPA, and 5 ml. M/10 tris(hydroxymethyl)aminomethane buffer (pH 7.6).

hydroxylamine was obtained after allowing it to condense with acetaldehyde under conditions comparable to those for the PNPA reaction. This would account for the mole for mole stoichiometry observed previously.

A similar explanation is not applicable to the lack of acetaldehyde noted when the cleavage was produced by GSH. Mercaptals, condensation products of mercaptans and aldehydes, are known to be quite stable to acids. Likewise, aldehydes have been shown to react with cysteine to give stable substituted thiazolidines (8, 9). If such compounds are formed in the reaction of GSH and PNPA, they may not be expected to release enough free aldehyde to be detected with the test used. The mixing of acetaldehyde and GSH under the conditions used in the cleavage experiment did not, however, interfere with the determination of acetaldehyde. Thus condensation with the thiol group, if it occurs, probably results from reaction of the GSH with PNPA without release of free acetaldehyde.

Irrespective of the mechanism of the reaction, it appears conclusive that acyl esters of *p*-nitrophenyl are unsuited as substrates for precise measurement of esterase or lipase activity. In addition to the apparent nonspecificity, other undesirable features of the method were noted during the course of this investigation. The use of substrate concentrations less than the K_m value[3] makes it absolutely necessary that the substrate concentrations be carefully adjusted in order to obtain comparable results from one test to another. The instability of the substrate often results in undesirably high rates of hydrolysis in water solution, particularly with high substrate concentrations. Also, since the pK of *p*-nitrophenol has been reported in the range of pH 7.00 to pH 7.15 (6) where the text mixtures are buffered, the pH at which the colorimeter readings are made is very critical (see Table I).

In conclusion, the use of acyl esters of *p*-nitrophenol as chromogenic substrates for esterase activity has been shown to be quite unsatisfactory and it might be well to view with some caution any enzyme assay method involving these and other similar chromogenic substrates.[4] Further investigation of the observed "reductive cleavage" may be of interest.

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[3] The K_m (Michaelis constant) is equal to the substrate concentration which results in a velocity of an enzyme reaction one-half of that theoretically obtainable with excess substrate.

[4] Salicylic acid esters have recently been suggested as substrates for the determination of choline esterase and lipase activity by Hofstee (3). However, acetylsalicylic acid, like PNPA, is cleaved by hydroxylamine and is probably not suitable as a substrate for enzyme measurements.

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THE USE OF ENZYME PREPARATIONS IN THE CRUDE FIBER DETERMINATION [1]

A. WEINSTOCK [2] AND G. HARVEY BENHAM [3]

ABSTRACT

A simple rapid method is described for the determination of crude fiber which is believed to be superior to the usual procedure based on acid and alkali digestion. The product under examination is subjected to the combined action of proteolytic and amylolytic enzymes present in the commercial preparation, Rhozyme S, and the residual material weighed.

In certain cases the fiber content may be determined by hydrolyzing the starch and determining the protein content in the residue. This simplified procedure gives results for the crude fiber content in good agreement with those obtained by complete enzymic hydrolysis.

The term "crude fiber" as denoted in this paper is the sum total of cellulose, pentosans and lignin remaining after an empirical treatment designed to remove protein, digestible carbohydrates and any small quantities of lipid material.

The determination of "crude fiber" is important to food chemists in detecting adulteration in various food products. For example, in examining spices the adulteration consists frequently in the addition of waste or refuse material derived from the spices themselves or from other food products. This material is usually the outer cellular layer or protective coating of the plant, and as such contains more of the hardened or lignified cells, resulting in higher values for crude fiber.

The biochemist is interested in the crude fiber content because of its commonly believed value as a measure of the indigestible portion of feedstuffs. The usefulness of this feed fraction as a general index is

[1] Manuscript received August 11, 1950. Contribution from Department of Biology, Illinois Institute of Technology, Chicago, Illinois.
[2] Present address: Armour Research Foundation, Chicago.
[3] Associate Professor of Biochemistry. Present address: Armour Research Foundation, Chicago.

conditioned by the source of the crude fiber and by the animal species to which the food is given.

The method of determining "crude fiber" adopted by the Association of Official Agricultural Chemists is the one devised by Henneberg (5) and is usually known as the Weende method. The name "Weende" is derived from the Weende Experimental Station in Göttingen, Germany, where Henneberg performed his experiments on the determination of crude fiber. The method, being a definitive one, does not represent a definite measure of a specific substance or group of substances. For this reason, it is subject to wide variation in the hands of different workers unless conditions are rigidly kept constant. Slight deviations in procedure, and particularly difficulties inherent in the method itself, e.g., filtration and transfer, cause lack of agreement. In 1947 Hunter (7) conducted a survey on the Weende method. Replies from 71 official and industrial laboratories showed many variations. A study of the information obtained from this survey revealed that deviations from the average were generally lacking in direction as the results of any one laboratory did not fall consistently on one side of the general average.

Far more serious, however, is the observation that the Weende method hydrolyzes to varying degrees cellulose, pentosans and lignin, constituents necessary in measuring indigestible portions of feedstuffs.

Haywood (4), using pure cotton cellulose, found that the acid digestion resulted in a 2.70% loss in weight, and that the alkali digestion resulted in a 17.06% loss in weight; a total of 19.76% cellulose digested. Norman (9) found that cellulose recovery by the Weende method is 60 to 80%, and lignin 4 to 67%. He determined that pentosans are digested during the 30 minute acid digestion period, and that lignin is extensively removed during the 30 minute alkali digestion period. Since the presence of lignin exercises a direct effect on the digestibility of the material, any empirical method should include all the lignin, and alkali treatment must be avoided. Norman suggested that acid hydrolysis may be a positive alternative method if a correction for protein is made.

Enzymic methods have been described before. In 1931 Remy (11) working with vegetables used pepsin, diastase and pancreatin in series at appropriate pH levels. The method called for incubation times of over eight days and results showed that about half of the indigestible residue was solubilized by the customary acid-alkali digestion method.

Williams and Olmstead (14) suggested a biochemical determination for the crude fiber in feces. They removed starch, protein and other primary substances by treating fecal matter with pancreatin in a buffer-bile salt solution, and checked their values by determining crude

fiber constituents in the residue. In 1936 Horwitt, Cowgill and Mendel (6) used a modified Remy procedure with spinach, substituting trypsin for pancreatin, and employing a clarase preparation. They obtained results three times as large as those given by the Weende method, but again the method involved an eight-day digestion period. Popov (10) and Woodson and Mackenzie (15) have used enzymic methods. The latter workers used pangestin for two separate 48 hour digestion periods. They reported values for a corn meal as follows:

	Crude Fiber, % Dry Basis
Horwitt method	8.76
Woodson and Mackenzie	8.23
Weende method	1.83

In this instance, it appears that the acid-alkali digestion has solubilized over three-quarters of the material not digested enzymatically.

The methods noted above are too time consuming to meet the needs of most laboratories. Recent development of potent commercial enzyme preparations such as Rhozyme S[4] and Polidase S[5] suggested that the use of such enzyme mixtures under optimum conditions would give values for the true indigestible matter in a shorter time. The present paper is concerned with the development of an enzymic method based on the use of these commercial enzyme preparations. The method is presented in order to stimulate further work to ascertain the correlations between actual digestibility and the results obtained by enzymic methods.

Development of Enzymic Method

Solubilization of Starch. The official method of solubilizing starch in cereals is to gelatinize 2 g. of the sample by boiling, to cool to 55°C., and to add a source of amylase such as malt or takadiastase. After one hour, it is re-boiled and the procedure repeated. The starch is solubilized to a negative iodine test in 2 hours. If, however, 1 g. of corn meal is boiled with 100 ml. of buffer for 15 minutes, cooled to 50°C. and immediately re-boiled for 5 minutes prior to any addition of enzyme, then one addition of 0.5 g. of Rhozyme S and incubation for one hour suffice to give a negative iodine test for starch in both residue and filtrate. If an autoclave is preferred, 10 minutes at 15 lbs. pressure suffices to gelatinize the starch completely.

Filtration Procedure. The filtration steps involved in the crude fiber determination have caused considerable inconvenience, and in many instances, variable results. This is especially true in the Weende acid-alkali method.

[4] Courtesy of Rohm and Haas Company, Philadelphia, Pennsylvania.
[5] Courtesy of Schwarz Laboratories, Inc., New York, New York.

Francis (3) conducted a series of collaborative determinations on crude fiber and the comments which he received emphasized the difficulty experienced in filtering, especially with samples that give colloidal dispersions with water. Neubert (8), using the Weende method, reported filtration times lasting from 1 to 24 hours. He suggested that the swelling of fibers during alkali digestion hindered the final filtration. Scharrer (12) experimented with glass crucibles and reported that they are not satisfactory because there is the possibility that small particles caught in the filter will not be properly ashed. He recommended the use of filter paper. Fincke (2) concurred with Scharrer that filter paper is preferable to the use of asbestos. Walker (13) studied the various filtration methods proposed by other investigators and arrived at the conclusion that the official method of the A.O.A.C., which specifies a porcelain Gooch crucible with an asbestos pad, is superior to other types. The authors have used the porcelain Gooch crucible with an asbestos pad in the Weende and enzymic method and find that it is not satisfactory. The Weende method being an arbitrary determination requires that the filtration time should not exceed 5 minutes. Slow filtration lengthens the time that the residue is in contact with the alkali, thereby resulting in an increased digestion time, and in deviations from the conditions set up for the Weende method to be reproducible.

The authors propose the use of a rapid filtration device as noted in Fig. 1. The alundum extraction thimble R.A. 98 is of medium porosity and is equivalent to the R.A. 98 alundum crucible used in many laboratories, and to the porcelain Gooch crucible suggested by the A.O.A.C. The thimble has the advantage of more filtration surface, thereby permitting rapid filtration and washing. The results obtained with the Gooch crucible and the alundum thimble on a sample that filtered rapidly were quite comparable.

Determination on Known Samples. To compare the crude fiber values obtained by the enzymic method and the Weende method, two samples of known crude fiber content were made up as follows:

Mixture A	Mixture B
0.10 g. crude fiber	0.15 g. crude fiber
1.50 g. corn starch	1.45 g. corn starch
0.40 g. corn gluten	0.40 g. corn gluten
2.00 g.	2.00 g.

The crude fiber component was obtained by ether extraction of dried corn fiber.[6] The residue was rendered free of all residual starch by enzymic hydrolysis and the weight used in making up the mixture was corrected for the small amount of protein present.

[6] Courtesy of Corn Products Refining Company, Argo, Illinois.

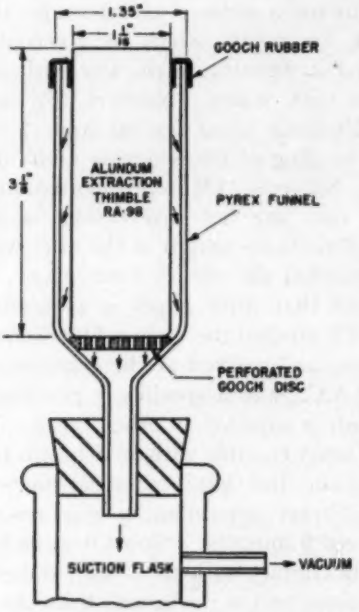

FIG. 1. Filtration device used in experimental work.

In the enzymic method, the samples were gelatinized by autoclaving in 100 ml. of 0.1 *M* potassium dihydrogen phosphate at pH 4.9. They were cooled to 52°C. and 0.9 g. Rhozyme S, or Polidase S, added. After 24 hours hydrolysis, the samples were filtered, dried, weighed, ashed, and re-weighed. The loss in weight upon ashing is the crude fiber. The Weende method was carried out as specified by the A.O.A.C. The results shown in Table I confirm the previous observations that the enzymic method gives values close to the actual crude fiber content, whereas the Weende method digests approximately

TABLE I

RECOVERY OF CRUDE FIBER WITH ENZYMIC AND WEENDE METHOD

Method	Enzyme	Crude Fiber Added	Crude Fiber Recovered	Loss
		g.	g.	%
Enzymic	Rhozyme S	0.100	0.108	—
Enzymic	Rhozyme S	0.150	0.150	—
Enzymic	Polidase S	0.100	0.110	—
Enzymic	Polidase S	0.150	0.150	—
Weende	—	0.150	0.034	77.0
Weende	—	0.150	0.032	77.4

77% of the crude fiber. The crude fiber showed 100% recovery by the enzymic method and 22.8% recovery by the Weende method, corresponding to an Enzymic/Weende ratio of 4.4.

Results and Discussion

In view of these preliminary findings, a study of the optimum conditions for enzymic digestion was made, using a large number of enzyme preparations, including Rhozyme S, papain, pangestin, Polidase S, Amprozyme, Takadiastase, and a new amylase-protease product furnished by the courtesy of Wallerstein Company.

The substrate chosen for these studies was a single large batch of Monarch Yellow Corn meal, from which all the samples were taken. The proximate analysis of this corn meal was as follows:

	%
Moisture	11.5
Ether extractibles	1.3
Ash	0.4
Starch (Diastase Method)	77.0
Protein (N × 6.25)	6.8

Consequently the crude fiber content is 3.0%. The method of Woodson and Mackenzie (15) using the enzyme Pangestin gave values approximating 3%, but the amount of insoluble enzyme present in the residue after hydrolysis resulted in very high blank values amounting to at least one-half of the total residue. The results were not readily reproducible.

Tests under the varying conditions shown in Table II indicated that Rhozyme S is a satisfactory enzyme preparation. It is completely water soluble at the optimum concentration used so that a blank determination is unnecessary. It has effective proteolytic activity at pH 4.9, so that it is unnecessary to alter the pH during hydrolysis. Starch is solubilized to a negative iodine test, both in the filtrate and residue, in one hour. Twenty-four hours at 50°C. suffices to hydrolyze the protein under these conditions. The average crude fiber value obtained under these conditions was 3.05% which is in close agreement with the proximate analysis. The Weende value on the same corn meal was 0.73% (Enzymic/Weende = 4.2). This indicates that 75% of the material which is being evaluated is digested and lost by the acid-alkali treatment.

A Proposed Enzymic Method for the Determination of Crude Fiber. Weigh 1 g. of ground sample into a 250 ml. beaker and add 100 ml. of 0.1 *M* potassium dihydrogen phosphate (pH 4.9). Autoclave the mixture for ten minutes at 15 lbs. pressure (or boil for 15 minutes) cool and reboil for five minutes. Cool the mixture to 50°C., add 0.45 g.

of Rhozyme S and mix well to dissolve the enzyme. Add to the mixture a trace of chloroform and 5 ml. of toluene, and incubate at 50°C. in a constant temperature water bath or incubating cabinet for 24 hours, replacing the toluene if necessary. Filter the contents through a weighed alundum extraction thimble R.A. 98. Wash the residue thoroughly with hot water and then ether, dry, weigh, ash and reweigh. The loss in weight is taken as crude fiber.

Alternative Rapid Method. A study of the results obtained under varied conditions for the crude fiber content of corn meal suggested that the residue left after short digestion times consisted of the crude fiber together with unsolubilized protein. Consequently, it is possible to use the following procedure where other circumstances permit:

Gelatinize a 1 g. sample as previously described, and hydrolyze the starch with Rhozyme S. At any convenient time after 1 hour, or when the iodine test is negative, filter the residue through tared filter paper, wash, dry and weigh. The residue in the filter paper consists of crude fiber plus unhydrolyzed protein (A). Drop the filter paper plus residue into a Kjeldahl flask and determine the protein content (N × 6.25) by the official method. The protein value (B) subtracted from the total original residue (A) yields the crude fiber.

Under many varying conditions of time and with many different enzymes, good values were obtained as shown in Table III.

TABLE II

EFFECT OF CONDITIONS ON PERCENTAGE CRUDE FIBER OBTAINED ENZYMATICALLY FOR DEGERMINATED CORN MEAL
(Digestion Temperature, 50°C.)

Enzyme	Sample Wt.	Digestion Time	Buffer	Residue "Crude Fiber"
	g.	*hours*	*pH*	%
0.1 g. Polidase S	2	5	4.9	11.5
0.5 g. Rhozyme S	1	1	In water	8.7
0.1 g. Rhozyme S	2	5	4.9	9.3
0.1 g. Rhozyme S	2	22	4.9	5.4
0.1 g. Polidase S	2	22	4.9	7.6
0.9 g. Rhozyme S	2	18	4.9	3.6
0.9 g. Polidase S	2	18	4.9	3.8
0.9 g. Rhozyme S	2	20	7.6	9.8
0.9 g. Polidase S	2	20	7.6	9.6
0.45 g. Rhozyme S	1	24	pH 4.9 (12 hrs.) changed to pH 7.5 (12 hrs.)	3.25
0.45 g. Rhozyme S	1	24	4.9 autoclave	3.05
0.9 g. Rhozyme S	2	24	4.9 boil	2.95
0.9 g. Rhozyme S	2	24	4.9 autoclave	3.14
0.45 g. Rhozyme S	1	24	4.9 autoclave	3.02
0.45 g. Rhozyme S	1	24	4.9 boil	3.10

TABLE III

RESULTS FOR CRUDE FIBRE OF DEGERMINATED CORN MEAL BY ALTERNATIVE RAPID METHOD

Enzyme	Hours	Total Residue A	Unhydrolyzed Protein B	Crude Fiber A–B
		%	%	%
Rhozyme S+papain	16	5.87	2.56	3.31
Polidase S	20	4.56	1.45	3.11
Rhozyme S	20	4.16	0.90	3.23
Rhozyme S	18	4.85	1.84	3.01
Rhozyme S	15	5.13	2.18	2.95
Rhozyme S+$MnSO_4$	15	5.17	2.18	2.99
Rhozyme S+NaCl	15	5.46	2.54	2.92
Rhozyme S	2	6.44	3.54	2.90

These results justify, within experimental error, a two hour incubation time followed by a protein determination, whereby a 3% crude fiber value is obtainable on the sample containing 3% of crude fiber.

However, in the 24 hour incubation time it is possible to digest all the protein under the conditions specified. The procedure is convenient as the operator can set up large numbers of samples which need essentially no attention during incubation.

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THE TREHALOSE CONTENT AND THE QUALITY OF ACTIVE DRY YEAST [1,2]

GLENN E. POLLOCK AND C. D. HOLMSTROM

ABSTRACT

Good gassing power of active dry yeast was associated with a high trehalose content. A study of fluctuations in total carbohydrate, trehalose, "glycogen," and mannan content of yeast showed that the trehalose content decreased to a very low value during budding and increased markedly during drying. The moisture range during the drying cycle at which the increase of trehalose virtually stopped was in the vicinity of 50–45% moisture.

The carbohydrates of yeast have been of interest to investigators since the initial work of Salkowski (8) on yeast mannan and glycogen. The procedures which he developed for mannan and glycogen have been used by later investigators with little modification. Subsequently, the disaccharide trehalose (11) was discovered to be a constituent of yeast. This was verified by workers such as Cori and Steiner (3) and later by Myrbäck and Oertenblad (5) who made exhaustive studies on the function of trehalose in the yeast cell. Their work was enlarged upon by Brandt (2) and recently Myrbäck and Elander (4) have demonstrated the formation of trehalose in yeast maceration juice. It was pointed out by Myrbäck (6) that this disaccharide may be present in bakers' yeast in quantities up to 10%. Recently, Payen (7) has reported that active dry yeast may contain up to 18% trehalose. He also noted that during the drying process, the glycogen content of the yeast decreased while the trehalose content increased. Richtmyer, Hudson, and Stewart (9), while investigating the trehalose content of various yeasts, devised an excellent method for the quick extraction of this disaccharide and its isolation in almost pure form with one crystallization. They also verified Payen's finding that active dry yeast contained large quantities of trehalose. Brandt (2) observed that the trehalose content of bakers' yeast decreased during budding whereas ripened yeast was high in trehalose. This fact coupled with the discoveries of Payen, and the results of some preliminary studies on active dry yeast carried out by the authors, led us to an investigation of the carbohydrate fluctuations of yeast during fermentation.

The objects of this study were to determine: (1) Whether a relation exists between the trehalose content of an active dry yeast and its

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[2] Contribution from Anheuser-Busch, Inc.

gassing power or quality; (2) The point at which the increase of trehalose during drying virtually ends; and (3) The fluctuations of the yeast carbohydrates during a complete fermentation. Growing methods are not the subject of the paper and growth curves are shown only in relation to trehalose metabolism.

Materials and Methods

Two series of pilot plant fermentations were carried out with a strain of *Saccharomyces cerevisiae* and samples were taken of the inoculum, three equal periods during the fermentation and the final cake. Samples were also taken at various intervals during the drying period. A Rotolouvre type dryer was used, although the drying apparatus is not nearly as important as the condition of the yeast to be dried. In addition to the carbohydrate analyses described later, routine phosphorus, total nitrogen (protein = total N × 6.25) and gassing tests were made on the yeasts. A dough fermentation test was also carried out on most of the yeasts.

Total Carbohydrates. A 0.3 g. sample (dry weight) was hydrolyzed in 20 ml. of 1.3 *N* hydrochloric acid solution in a boiling water bath for 4 hours, transferred to a 100 ml. volumetric flask and neutralized with 15% sodium hydroxide solution to a phenolphthalein end point. Zinc sulfate solution (5 ml. of 20%) was added and neutralized with saturated barium hydroxide solution to a phenolphthalein end point. After making up to volume with distilled water, the Munson-Walker gravimetric sugar procedure (1) was used to determine the total carbohydrate as glucose equivalent.

Trehalose. The method of Stewart, Richtmyer, and Hudson (9) was used with certain modifications. A 1 g. sample (dry weight) was extracted at 50°C. for one half hour with 25 ml. of ethanol adjusted so that the final alcohol concentration would be 75% (v/v). The extract was not passed through ion exchange resin. Clarification and hydrolysis of a 50 ml. aliquot of the extract were carried out as under the total carbohydrate procedure. Analysis of the hydrolysate for reducing sugar was made by the Munson-Walker procedure. The trehalose was calculated as the glucose equivalent.

Mannan. The procedure used was essentially that described by Stockhausen and Silbereisen (10) with reduction for use with smaller samples (5 g.). Mannan was calculated as the glucose equivalent.

Take a 5 g. sample of press yeast (wet weight) and place in a 20 × 200 mm. test tube. Add 20 ml. of approximately 65% potassium hydroxide solution to the test tube. Heat in a boiling water bath for 4 hours. While still hot, transfer to a 100 ml. volumetric flask using a minimum of hot distilled water. Neutralize with glacial

acetic acid till slightly pink to phenolphthalein. This precipitates much of the protein. Cool to room temperature and make up to volume. Filter through No. 41 Whatman filter paper and using a volumetric pipette, transfer 25 ml. of the filtrate to a 100 ml. beaker. To this solution add 25 ml. of mixed Fehling's solution and stir vigorously with a small glass rod in order to coagulate the copper mannan precipitate. Let it stand over night and filter the next day, using a small funnel so that a minimum of chilled water is used for washing. After washing till little or no more color appears in the filtrate, dissolve the precipitate in 1.3 *N* hydrochloric acid solution, using small increments of the acid to effect solution of the precipitate. Use a total of 55 ml. of acid for this operation. The filtrate is collected in a 100 ml. volumetric flask and hydrolyzed in a boiling water bath for 4 hours. Remove and cool to room temperature. Neutralize the acid with 20% sodium hydroxide solution till slightly pink to phenolphthalein. Dilute to mark and mix. If a small protein flocculate appears, filter it off. Weigh out two 0.1000 g. samples of glucose and place them in 400 ml. beakers. To one add 50 ml. of the hydrolyzed mannan and to the other add 50 ml. of distilled water. To each of the beakers add 50 ml. of mixed Fehling's solution. Carry out the reduction as specified by Munson and Walker. The weight of the blank glucose is subtracted from the weight of the mannan plus the glucose to give the cuprous oxide due to the mannan.

Glycogen. "Glycogen" was estimated in terms of its glucose equivalent by difference. Glucose equivalent of total carbohydrate minus trehalose and mannan as glucose equivalents equals "glycogen" as glucose equivalent. This procedure was followed because no adequate methods are available for the accurate determination of this fraction of yeast, and since it is doubtful that glycogen is the only glucose polymer obtained under the conditions specified for the procedures which are available.

Dough Fermentation Test. A mix is prepared by adding 325 g. of patent spring wheat flour and 6.5 g. of shortening to a mixing bowl, followed in turn by 6.5 g. salt and 10.0 g. sucrose. To this is added 4.4 g. of yeast which has been soaked in 50 ml. of water at 43.5°C. (110°F.) for 10 minutes. The container which held the yeast is then rinsed out with water until a total of 200 ml. of water has been added to the mix. These ingredients are mixed thoroughly and the resulting dough is removed from the mixer, rounded and placed in a calibrated, 1,500 ml. Chidlow jar. Carefully expel all air pockets with the use of a hollow glass tube and then press the dough down until the surface is practically level. Place into an 29.5°C. (85°F.) fermentation cabinet and record the time. Allow the dough to rise to a volume of 1,300 cc.

The time elapsed to the first punch is calculated as 60% of the total fermentation time.

Moisture. Moisture was determined by the loss in weight of a 3–5 g. sample of yeast dried in an air oven at 105°C. for 16 hours.

All analyses given in the following tables and graphs are on a dry substance basis.

Results

Shown in Table I are the results of analyses on three good and three poor active dry yeasts as evaluated by gas production.

Yeasts 77, 74 and commercial 1 are considered to be good yeasts and have a high trehalose content while 78, 76 and 70 are poor yeasts and have a low trehalose content. The latter two were so poor in

TABLE I

SURVEY ANALYSES OF SEVERAL ACTIVE DRY YEASTS WHICH INDICATE A POSSIBLE ASSOCIATION OF TREHALOSE CONTENT AND GASSING POWER

Yeast	Trehalose	Mannan	Glycogen	Total Carbohydrate	Protein	Phosphorus[1]	Initial Gas Production
	%	%	%	%	%	%	*mm. Hg*
77	15.2	10.2	12.6	38.0	39.7	1.6	165
74	17.8	11.3	13.0	42.1	39.0	1.5	161
Commercial-1	18.6	9.6	12.5	40.7	—	—	163
78	12.3	11.7	13.3	37.2	40.7	1.8	112
76	10.9	11.2	14.1	36.2	43.3	2.2	—
70	11.3	10.1	12.9	34.2	45.8	1.8	—

[1] Expressed as P_2O_5.

baking tests that gassing values were not determined. This apparent agreement between trehalose content and gassing power led us to investigate further the carbohydrates of yeast.

Five pilot plant fermentations were made in order to follow the fluctuations of the carbohydrates during the fermentations. Samples were taken as described earlier in the paper. Two different methods of growing the yeasts were used. These methods will not be described aside from the general budding curves which are given in Fig. 1. In Procedure I, the budding was initiated early and a high percentage was maintained throughout the greatest part of the fermentation. In Procedure II, the yeast was conditioned for a few hours, then budding was begun and concentrated in a short period, followed by another conditioning period.

The results of analyses of the press cake of these fermentations are given in Table II.

Trehalose dropped markedly during the budding periods of both growing procedures. The total carbohydrate increased steadily in

Procedure II; in Procedure I after an initial drop it increased steadily also. Mannan and glycogen values showed a steady increase throughout the fermentations except with the latter in Procedure I, where there was an initial drop. The total carbohydrate, glycogen and mannan, in general, increased steadily throughout the fermentations even when the trehalose had decreased to a very low value.

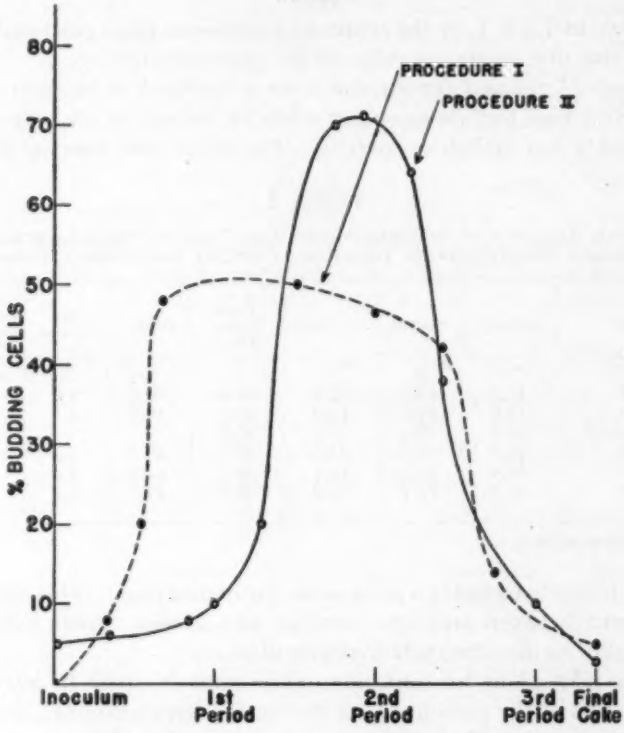

FIG. 1. General budding curves showing peak budding periods. Procedure I, budding initiated early; Procedure II, budding delayed.

These five yeasts were then dried and analyses of trehalose and moisture were made on samples taken at various stages of the drying period. The results, given in Fig. 2, verify the work of Payen (7) in which he claims that trehalose increases during the drying period. The increase in the trehalose content of the yeast seems to stop or slow down very abruptly at a moisture content of from 50 to 45%. This demonstrates the apparent importance of water to the production of trehalose during the drying period. The possible significance of this will be discussed later.

TABLE II

Carbohydrate Fluctuations in Yeast Press Cake at 5 Stages of the Fermentation Process [1]

% Trehalose

Fermentation No.	Inoculum	Period			
		1st	2nd	3rd	Final Cake
30*	6.5	—	2.6	6.5	7.8
32*	7.3	4.8	3.9	5.3	8.1
34*	7.7	4.8	4.1	6.5	11.0
31**	7.3	0.6	2.3	3.9	6.6
33**	4.4	1.7	1.0	1.9	10.1

% Mannan

Fermentation No.	Inoculum	1st	2nd	3rd	Final Cake
30*	9.4	—	13.9	15.0	13.4
32*	9.4	10.2	10.9	11.5	11.7
34*	10.0	10.5	11.4	11.6	11.6
31**	7.7	8.5	8.8	8.1	10.5
33**	10.3	11.5	13.4	13.9	12.4

% Glycogen

Fermentation No.	Inoculum	1st	2nd	3rd	Final Cake
30*	12.8	—	12.7	14.0	18.8
32*	10.2	15.7	16.3	17.2	20.0
34*	10.7	14.1	16.2	17.6	16.7
31**	14.4	11.0	14.7	16.9	17.6
33**	18.7	16.2	13.4	15.9	17.2

% Total Carbohydrate

Fermentation No.	Inoculum	1st	2d	3rd	Final Cake
30*	28.7	—	29.2	35.5	40.0
32*	26.9	30.7	31.1	34.0	39.8
34*	28.4	29.4	31.7	35.7	39.3
31**	29.4	20.1	25.8	28.9	34.7
33**	33.4	29.4	27.8	31.7	39.7

* Procedure 2.
** Procedure 1.
[1] The nitrogen and phosphorus contents of the yeast are as follows:

Fermentation No.	Final Cake	
	Total N	Phosphorus‡
32	7.32	1.58
34	7.44	1.91
31	7.32	1.49
33	6.33	1.91

‡ Expressed as P_2O_5.

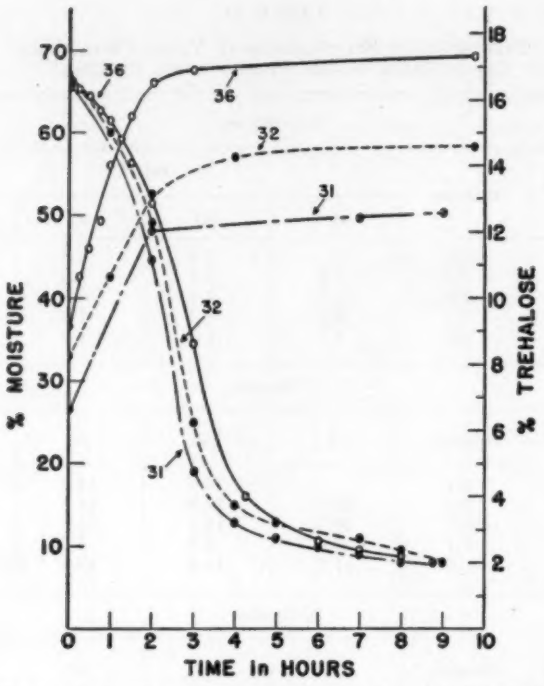

FIG. 2. A comparison of trehalose and moisture changes during drying.

In Table III analytical data are given for all of the active dry yeast samples on which trehalose, gassing analyses and dough fermentation tests have been made in an attempt to determine whether a positive relation exists between high trehalose content and high gassing power.

It may be said, with little reservation, that if an active dry yeast contains 15% trehalose or more, it will have good gassing and baking potentialities. Yeast 33 is the only exception to this generalization.

TABLE III

ASSOCIATION OF TREHALOSE CONTENT AND QUALITY OF ACTIVE DRY YEAST

Yeast No.	Ferm.	Gas	Trehalose	Yeast No.	Ferm.	Gas	Trehalose
	min.	*mm. Hg*	%		*min.*	*mm. Hg*	%
34	93	177	15.5	76	110	<102	10.9
36	98	173	17.3	70	142	<102	11.3
77	96	165	15.2	30	—	<102	11.4
Commercial-1	—	164	20.2	31	160	102	12.5
Commercial-2	88	163	18.6	78	123	112	12.3
74	102	161	17.8	33	123	120	17.5
18	—	157	17.4	19	—	135	14.1
32	—	146	14.5				

Discussion

The data presented in this paper show a positive association between the trehalose content of active dry yeast and its gassing and baking ability. The data further indicate that the moisture content of the yeast during the drying period seems to be a limiting factor on the production of trehalose during this time. It was shown in Fig. 2 that the greatest increase in trehalose comes during the time interval when the yeast contains over 50% moisture. After this point is reached, very little trehalose production occurs and in some cases it virtually stops. This observation seems to indicate that the intracellular water may be concerned in this trehalose increase. How closely the water content of yeast is tied up with trehalose metabolism is not very clear at this time. One of the authors (C. D. H.) has consistently found yeast in which a high percentage of budding is occurring to contain 70% or more moisture. This is quite a contrast to the final active dry press cake, which usually contains 65–67% moisture. It will be noted from Table II that during the budding period the trehalose content drops to a very low value. Whether this trehalose drop and the usual high moisture content of the yeast at this point are connected is questionable, since one may explain this high moisture value in other ways. The authors, however, feel that the loss of water during the drying period serves as the activator which sets off the reaction that synthesizes trehalose, possibly at the expense of glycogen.

Acknowledgments

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THE RELATION BETWEEN ALCOHOL-SOLUBLE AND TOTAL NITROGEN CONTENTS OF OATS [1]

K. J. FREY [2]

ABSTRACT

Of the oat varieties analyzed in this study Huron was lowest and C.I. 5298 highest in both total nitrogen and alcohol-soluble nitrogen. The alcohol soluble nitrogen content of the low and high protein varieties of oats was essentially constant at 18% of the total nitrogen.

The alcohol soluble protein of the cereal grains, especially of corn, wheat, and oats, has been shown to be of poor nutritive value (6). This poor quality results from a deficiency in one or two (lysine and tryptophan) of the essential amino acids (6, 7). Oat protein is generally considered to be equal or slightly better in quality to that of corn (6, 8). Csonka found that about 16% of the oat protein was soluble in 70% ethanol. In corn, Showalter and Carr (9) found that the proportion of the protein composed of zein was greater in Illinois high-, than in Illinois low-protein corn. Frey (3) reported that as the protein percentage of the corn increased, the zein became a larger proportion of the total protein. Thus, as the protein percentage in corn was increased, the corn protein became poorer in nutritive value.

The objective of the present study was to determine, as in the case of corn, whether the total nitrogen-alcohol soluble nitrogen ratio, varied in the grain from different varieties and strains of oats under investigation at the Michigan Agricultural Experiment Station.

Materials and Methods

The strains and varieties of oats used in this study (Table 1) were grown at East Lansing, Michigan in 1947 and 1949. A majority of the varieties were included both years. The seed of each variety from two replications was composited and thoroughly mixed and a 100 g. sample was removed for analysis. The samples were ground to pass through a 40-mesh sieve.

Total nitrogen was determined by the Kjeldahl-Gunning-Arnold method and alcohol-soluble nitrogen was determined with the method described by Frey (3) for zein.

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Results and Discussion

The total and alcohol soluble nitrogen percentages for the 31 samples are given in Table I. It is obvious from the data that there is a positive relationship between total nitrogen and alcohol soluble nitrogen.

The alcohol-soluble/total nitrogen ratios (Table I) range from 0.16 to 0.20. This suggests that there is no difference in the high

TABLE I

THE PER CENT OF TOTAL AND ALCOHOL SOLUBLE NITROGEN IN OAT VARIETIES GROWN AT EAST LANSING IN 1947 AND 1949

Variety	1947			1949		
	Total N	Alc-Sol N	A/T[1]	Total N	Alc-Sol N	A/T[1]
	%	%		%	%	
Ajax	—	—	—	2.42	0.43	0.18
Andrew	2.04	0.42	0.20	—	—	—
Beaver	2.01	0.37	0.18	2.66	0.46	0.18
Bonda	2.14	0.38	0.18	2.66	0.45	0.17
Clinton	1.87	0.34	0.18	2.54	0.48	0.19
Colo	1.71	0.32	0.19	2.56	0.46	0.18
Eaton	2.14	0.37	0.17	2.46	0.46	0.19
Forvic	1.98	0.32	0.16	—	—	—
Huron	1.49	0.29	0.19	2.21	0.37	0.17
Kent	—	—	—	2.77	0.46	0.17
Mindo	1.87	0.35	0.19	2.40	0.40	0.17
Mohawk	1.90	0.34	0.18	2.45	0.43	0.18
Overland	1.74	0.32	0.18	—	—	—
Shelby	—	—	—	2.26	0.43	0.19
Vicland	1.98	0.34	0.17	—	—	—
Volverine	1.90	0.34	0.18	2.54	0.40	0.16
Worthy	1.79	0.34	0.19	2.27	0.38	0.17
C.I. 3656[2]	2.32	0.42	0.18	2.56	0.51	0.20
C.I. 5298[3]	2.53	0.48	0.19	2.69	0.51	0.19
Mean	1.97	0.35	0.18	2.50	0.45	0.18

[1] A/T means ratio of alcohol-soluble nitrogen to total nitrogen.
[2] Selection from a Bond-Iogold cross.
[3] Michigan No. 44720, a selection from a Bond-Anthony cross.

and low nitrogen samples with respect to the proportion of the total nitrogen composed of the alcohol-soluble fraction. To determine whether the ratio of alcohol soluble to total nitrogen could be considered constant over the whole range of nitrogen percentages a statistic recently reported by Frey (3) was used. This consists of calculating the "b" value for the equation $Y = aX^b$, in which Y and X represent total nitrogen and alcohol soluble nitrogen, respectively, and a and b are constants calculated from the data. The regression, b, measures the constancy of the ratio of X to Y when both X and Y are increasing; hence, if b is equal to unity, X (in this case alcohol

soluble nitrogen) is a constant proportion of Y (in this case total nitrogen).

The "b" value was found to be 0.94 and 1.06 for the 1947 and 1949 data, respectively. Neither of these regression coefficients was significantly different from unity or from one another, and the two regression lines plotted from them coincided so closely that the sample values for the two years were pooled. The regression value for the combined data was 0.95, and when this was tested against 1.0, a non-significant t value of 0.64 resulted. Insofar as the varieties of oats used in this study are quite representative, it is evident that the alcohol-soluble nitrogen remains a constant proportion of total nitrogen in the oat grain regardless of the total nitrogen percentage.

Furthermore, it is also shown in Table I that no variety of oats had a high-or a low-ratio of alcohol soluble to total nitrogen in both years. For instance, Wolverine & C.I. 5298 have the extreme ratios of 0.16 and 0.20, respectively, in 1949, but in 1947 their ratios were both 0.18. Also the average ratio for the varieties common to both years was 0.18 in each while the total nitrogen percentage in 1949 was 0.53 higher than in 1947.

If one were to use the alcohol-soluble/total nitrogen ratio in the oat grain as a criterion of nutritive value of the oat protein, the data presented in this study indicate that regardless of the level of nitrogen percentage in the oats, the total protein does not change in its nutritive value. This is different than the comparable relationship in corn where Showalter and Carr (9), and later Frey (3) showed that as the total protein percentage increased, the zein fraction became a greater proportion of the total protein. Thus, the total protein of high protein corn was less nutritionally balanced than that of low protein corn.

These data indicate that the scheme used by Frey *et al.* (4) in an attempt to improve the protein of corn would not be feasible with oats. The most logical approach would be simply to select oat varieties with a higher nitrogen percentage. In using this method of selection it must be kept in mind that there tends to be a negative correlation between yield per acre of a cereal crop and the percent of total nitrogen in the grain (2, 5). Consequently, the nitrogen harvested per acre of crop does not increase in proportion to the rise in protein percentage.

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BOOK REVIEWS

Malt Amylases and Their Action on Starch. Prepared by Muntona, Ltd., Bedford, and Edward Fison, Ltd., Ipswich. 1951. 18 pp. Gratis.

This is the first of a series of Monographs on Malt and Malt Products prepared by these two English malting companies as a contribution to the British Festival Year.

This monograph gives a summary of the existing knowledge of malt enzymes, their actions on starch, the factors affecting such actions, and the methods of measuring amylase activity. The author although unknown has managed to present very lucidly all of the salient facts concerning our present knowledge of malt amylases and their actions. There is no general bibliography but only a brief one on methods of amylase measurement.

This monograph is recommended as an introduction to malt amylases for students and for technical and supervisory personnel in the malting, brewing, distilling, and cereal industries.

SUTTON REDFERN
The Fleischmann Laboratories
Standard Brands, Inc.
New York 51, N. Y.

"Stoffwechsel und Ernährung." By Konrad Lang and Otto F. Ranke. 1950. Published by Springer-Verlag, Berlin. 289 pages.

Within the framework of the more extensive "Lehrbuch der Physiologie" edited by the late W. Trendelenburg and by E. Schütz, the biochemist Lang and the physiologist Ranke have joined their efforts to write a book on metabolism and nutrition. The subject matter has been divided by the authors into two main sections, one covering 89 pages deals with energy relationships, the other covering 177 pages describes mainly the function of elements and compounds in nutrition. The most important individual chapters are devoted to the following topics: determination of the energy balance; basal metabolism; regulation of energy metabolism; isodynamics and specific dynamic action; energy metabolism of work; energy metabolism under conditions of decreased energy intake; decrease of body substance during insufficient energy intake; recovery after inanition; utilization of food substances; the importance of carbohydrates, of fats and of proteins respectively in nutrition; the importance of mineral elements in nutrition; the importance of trace elements in nutrition; the importance of vitamins in nutrition. These main chapters are followed by shorter but pertinent discussions of: nutritionally-induced liver damage; nutrition, reproduction and life-span; bread; newer sources of protein; vegetarianism and the consumption of raw food. In view of the experiences in Europe during World War II, it is not surprising that the authors have devoted considerable space to a discussion of the effects of inanition on the human organism. In this discussion extensive reference is made to the classical experiments of A. Keys and his associates conducted at the University of Minnesota. It is regrettable that prior to the preparation of their manuscript Lang and Ranke did not have access to A. Keys' book on "Biology of Human Starvation" which was published approximately at the same time as the book under review.

Throughout the text, the discussion is focused primarily on problems of human nutrition, although evidence provided by experiments with animals is freely cited particularly in the section dealing with the function of various nutrients. It is quite evident that a discussion of intermediary metabolism of nutrients and of the synthetic capabilities and limitations of the animal organism have been purposely omitted by the authors. Reference to some of these reactions would however have been profitable and could have been included in appropriate places without great expansion of the text. Thus, in the discussion of cholesterol as a dietary factor and its relation to atherosclerosis, the demonstrated ability of some species to synthesize cholesterol

from products of carbohydrate or fat metabolism, would have thrown a more realistic light on the problem. In general, however, the reviewer considers the choice, the presentation of various phases of the subject matter, and the balance between them highly satisfactory. Particularly impressive is the careful documentation of the statements through an extensive, up-to-date bibliography of international scope. The most pertinent points of the discussion are usually emphasized in tabular form. The 135 tables and 35 figures have been carefully selected and many of them have been compiled by the authors from available data. These tables constitute one of the most valuable features of this book which contains a surprisingly large amount of information for its size.

Relatively few errors have been noted (the formula for thiamine on p. 217 requires correction; the metabolite of pyridoxine is given as N′-methyl-2-pyridone-3-carboxylamide instead of the 6-pyridone). The printing and binding are very satisfactory. This book is a valuable addition to the library of any laboratory or institute concerned with problems of human nutrition.

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Industrial Oil and Fat Products (second edition). By Alton E. Bailey. 992 pp. Interscience Publishers, Inc., New York. 1951. Price $15.00.

The first edition of this book, published in 1945, admirably filled the need at that time for a good general text on the technology of fats and oils. Since then, marked advances in knowledge of the chemistry of fats and oils have been made, accompanied by many new technological developments. The second edition is extensively revised and rewritten to give a good coverage of the recent advances. The format is essentially the same as in the first edition except that the size of the text has been increased by approximately 200 pages. In addition, the author has made various improvements in what was already a very well organized and systematically arranged book.

The volume is divided into four sections and twenty-three chapters. In section A, The Nature of Fats and Oils, one of the chapters dealing briefly with reactions of fats and fatty acids has been expanded to good advantage. In section B, Raw Materials for Oil and Fat Products, a chapter on the production and consumption of primary fats and oils has been deleted, partly, according to the author, "because of an inadequacy of recent world-wide statistical data." In section C, chapters on the industrial utilization of fats and oils; soap and other surface active agents; and paints, varnishes, and related products, have been expanded considerably by the addition of both old and new information, and some additions have also been made in chapters on plastic shortening agents, and butter and margarine.

The most extensive changes have been made in section D, Unit Processes in Oil and Fat Technology. Here the handling, storing, and grading of oil-bearing materials has been made the subject of a separate chapter, and chapters dealing with extraction; refining and bleaching; hydrogenation; fat-splitting, esterification, and interesterification; and polymerization, isomerization, and related processes, have been extensively revised and expanded.

Other changes that will facilitate the use of this volume are the listing of all chapter subheadings in the table of contents, and a more comprehensive listing of subject matter in the subject index.

The volume is well balanced in that it treats various raw materials, uses, and processes in proportion to their technological importance. For college students, research chemists, and specialists in the field of fat and oil products, it is not possible to find a better general reference work on fat and oil technology.

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The Chemistry and Technology of Food and Food Products. Edited by Morris B. Jacobs. 1951. Second edition. Volume I. v + 832 pp. Interscience Publishers, Inc., New York. Price $12.00.

An embryonic food technologist examining this book for the first time might well be discouraged by the vast fund of knowledge expected of him in his chosen field. Yet there is no escaping the fact that the tremendous complexity of the food industry of today has created problems which can only be solved by an intelligent application of principles which encompass almost every known branch of scientific endeavor. "The Chemistry and Technology of Food and Food Products" with its three volumes and 52 chapters represents the collaborative effort of 39 experts in the fields of chemistry, chemical engineering, biochemistry, bacteriology, sanitary engineering, public health, food inspection and entomology. Volume I, with which this review is concerned, is divided into three parts. The first part deals with such fundamental aspects as physical chemistry, carbohydrates, lipids, proteins, minerals, vitamins, enzymes, metabolism, and food spoilage; in part two, unit operations and processes applicable to most foods are described; part three deals with the maintenance of sanitary and quality control.

Each chapter comprising part one treats its subject matter in a very comprehensive manner, and constitutes a source of reference comparable to and in some cases exceeding that found in standard textbooks. Chapter 2 (Jacobs) on physical chemistry is particularly commendable because the principles of physical chemistry are emphasized in relation to their application in the food industry. Unfortunately some of the subsequent chapters do not always succeed in closing the gaps that sometimes exist between fundamental and applied knowledge. To illustrate, the following examples have been chosen at random: In chapter 5 (Thomas), dealing with proteins and amino acids, no mention is made of the fact that amino acid analyses *per se* may frequently be misleading as an index of protein quality particularly in processed foods where the "browning reaction" is known to interfere with the biological availability of some amino acids. The section on "antienzymes" in chapter 6 (Jacobs) has omitted the trypsin inhibitor of raw soybean as an example but has included instead anticatalase and antipepsin produced by "immunizing" rabbits with catalase and pepsin respectively. Chapter 7 (Rosenberg), which includes an excellent treatise on hormones, fails to mention the use of hormonal substances such as stilbestrol and thyroprotein in the feeding of farm animals. In the same chapter, more emphasis might have been placed on the loss of nutrients incurred during the processing of foods.

Part two, which consists of two chapters (Gaver) on unit operations and processes, appears to have relied rather heavily on a few selected references, particularly Cruess' "Commercial Fruit and Vegetable Products." Part three dealing as it does with sanitary and quality control contains information which is extremely useful and which is generally difficult to find except in scattered sources.

A few typographical errors were encountered: "argenase" for arginase (p. 149); chapter 11 (Halvorson) had at least three errors in the numbering of footnotes (pp. 417, 434, 436).

By bringing together into a single text the many fundamental and specialized phases of food production, this book provides a broad and constructive treatment of food technology. As such, it should be of interest and value to teachers, students, food manufacturers, and those concerned with the official supervision and inspection of food supplies.

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Cereal Chemistry

EDITORIAL POLICY

Cereal Chemistry publishes scientific papers dealing with raw materials, processes, or products of the cereal industries, or with analytical procedures, technological tests, or fundamental research, related thereto. Papers must be based on original investigations, not previously described elsewhere, which make a definite contribution to existing knowledge.

Cereal Chemistry gives preference to suitable papers presented at the Annual Meeting of the American Association of Cereal Chemists, or submitted directly by members of the Association. When space permits, papers are accepted from other scientists throughout the world.

The papers must be written in English and must be clear, concise, and styled for *Cereal Chemistry*.

Manuscripts for publication should be sent to the Editor in Chief. Advertising rates may be secured from and subscriptions placed with the Managing Editor, University Farm, St. Paul 1, Minnesota. Subscription rates, $9.00 per year. Foreign postage, 50 cents extra. Single copies, $2.00; foreign, $2.10.

SUGGESTIONS TO AUTHORS

General. Authors will find the last volume of *Cereal Chemistry* a useful guide to acceptable arrangements and styling of papers. "On Writing Scientific Papers for Cereal Chemistry" (*Trans. Am. Assoc. Cereal Chem.* **6**: 1–22. 1948) amplifies the following notes.

Authors should submit two copies of the manuscript, typed double spaced with wide margins on 8½ by 11 inch white paper, and all original drawings or photographs for figures. If possible, one set of photographs of figures should also be submitted. Originals can then be held to prevent damage, and the photographs can be sent to reviewers.

Editorial Style. A.A.C.C. publications are edited in accordance with *A Manual of Style*, University of Chicago Press, and *Webster's Dictionary*. A few points which authors often treat wrongly are listed below:

Use names, not formulas, for text references to chemical compounds. Use plural verbs with quantities (6.9 g. were). Figures are used before unit abbreviations (3 ml.), and % rather than "per cent" is used following figures. All units are abbreviated and followed by periods, except units of time, which are spelled out. Repeat the degree sign (5°–10° C.). Place 0 before the decimal point for correlation coefficients ($r = 0.95$). Use * to mark statistics that exceed the 5% level and ** for those that exceed the 1% level; footnotes explaining this convention are no longer required. Type fractions on one line if possible, e.g., $A/(B+C)$. Use lower case for farinograph, mixogram, etc., unless used with a proper name, i.e., Brabender Farinograph. When in doubt about a point that occurs frequently, consult the Style Manual or the Dictionary.

For more detailed information on manuscript preparation see *Cereal Chem.* **27**: 519–520 (1950).

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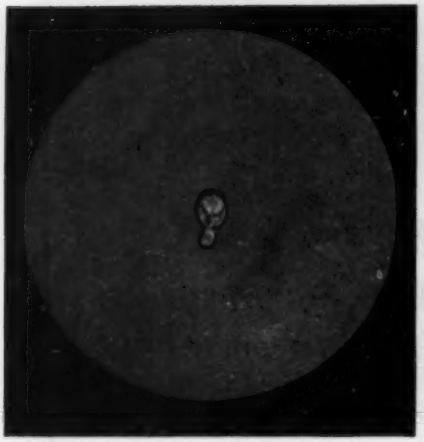

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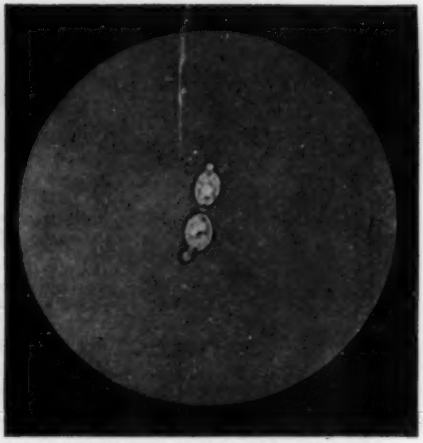

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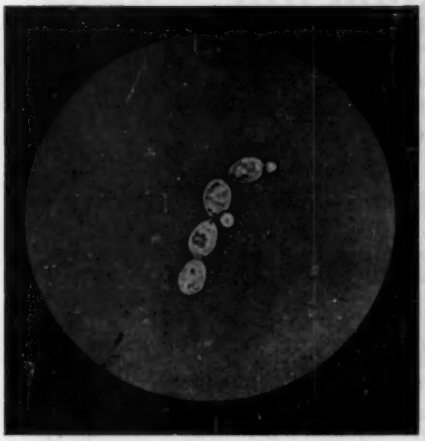

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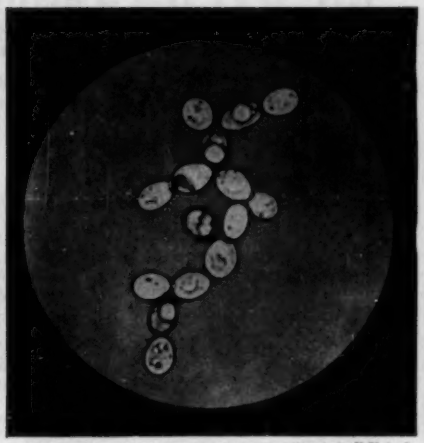

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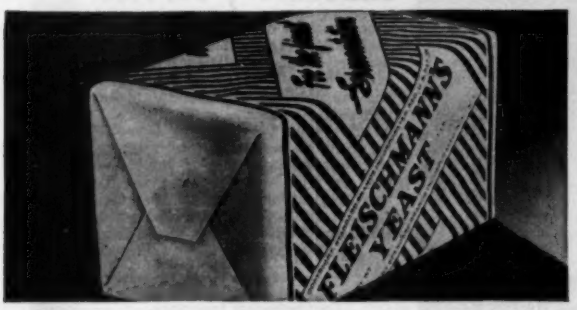

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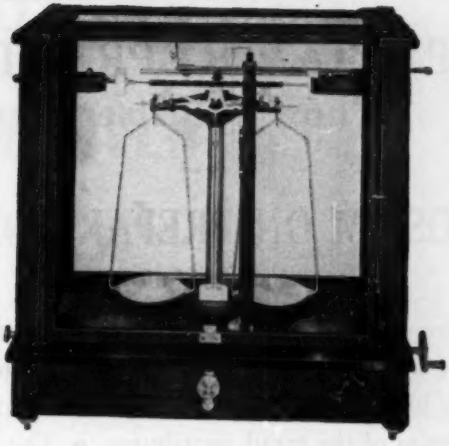

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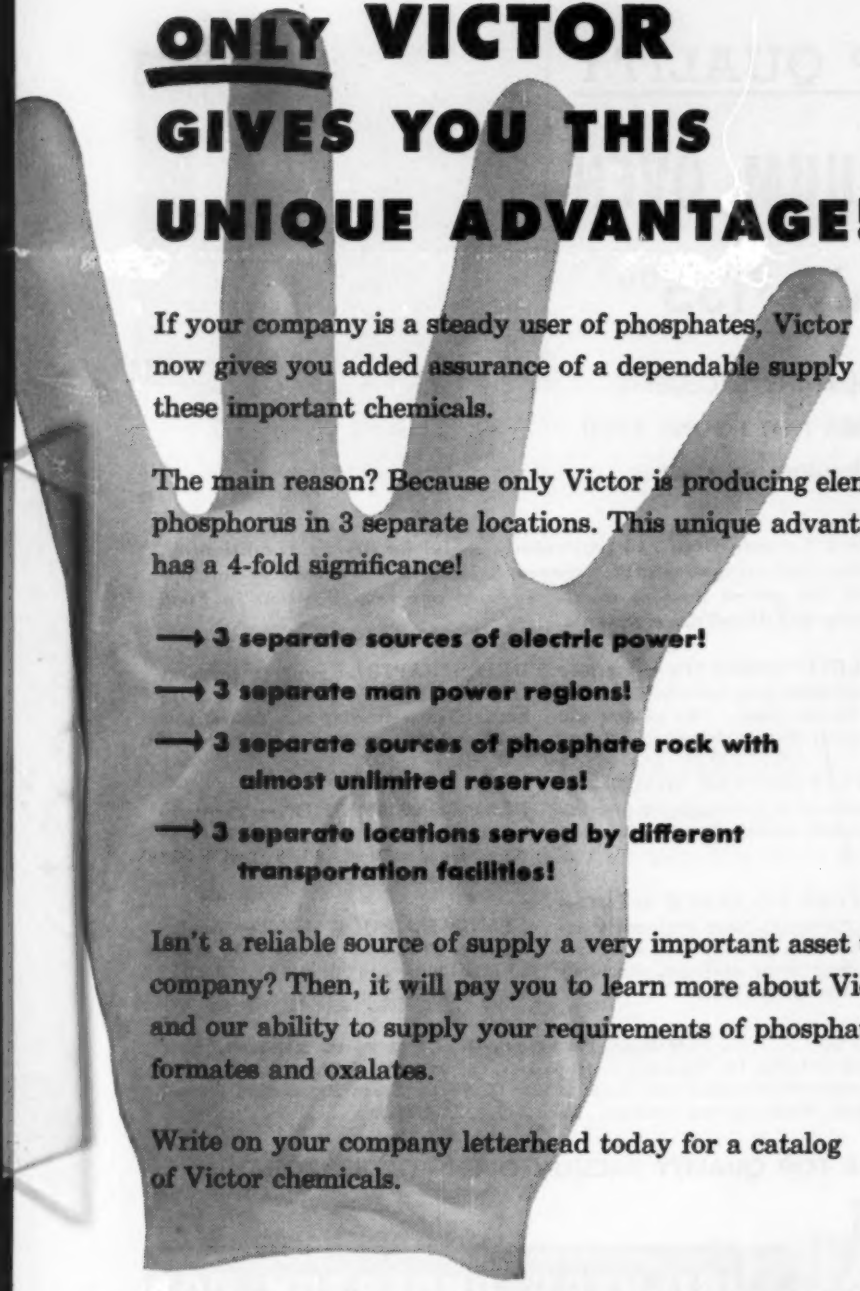

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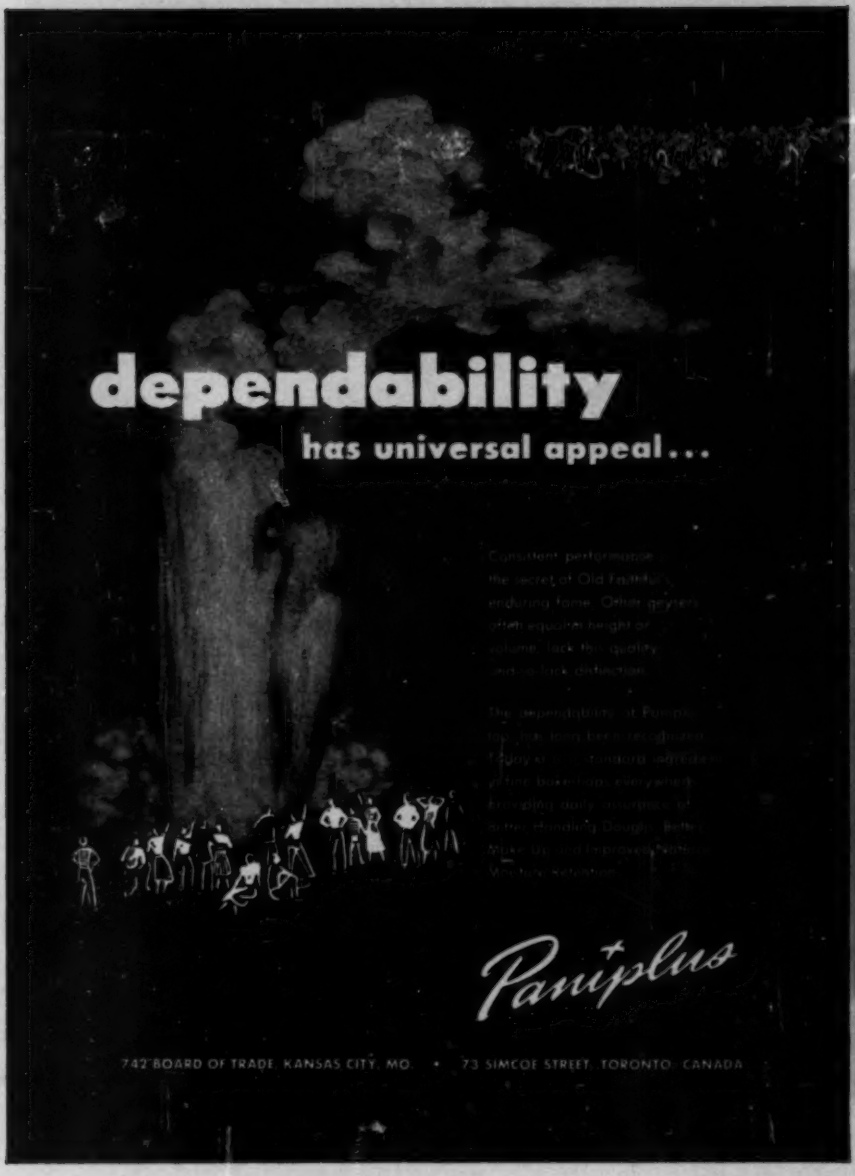
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